MOLECULAR CHARACTERIZATION OF MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA*

Abdelkodose Mohammed Hussen Abdulla

THESIS SUBMITTED IN FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

FACULTY OF MEDICINE
UNIVERSITY OF MALAYA
KUALA LUMPUR, MALAYSIA

2012

UNIVERSITI MALAYA

ORIGINAL LITERARY WORK DECLARATION

Name of Candidate: Abdelkodose Mohammed Hussen Abdulla

(Passport No: (03008193)

Registration/Matric No: MHA090012

Name of Degree: PhD

Title of Thesis: "MOLECULAR CHARACTERIZATION OF MULTIDRUG RESISTANT *PSEUDOMONAS AERUGINOSA*"

Field of Study: Bacteriology

I do solemnly and sincerely declare that:

(1) I am the sole author/writer of this Work;
(2) This Work is original;
(3) Any use of any work in which copyright exists was done by way of fair dealing and for permitted purposes and any excerpt or extract from, or reference to or reproduction of any copyright work has been disclosed expressly and sufficiently and the title of the Work and its authorship have been acknowledged in this work;
(4) I do not have any actual knowledge nor do I ought reasonably to know that the making of this work constitutes an infringement of any copyright work;
(5) I hereby assign all and every rights in the copyright to this work to the University of Malaya ("UM"), who henceforth shall be owner of the copyright in this work and that any reproduction or use in any form or by any means whatsoever is prohibited without the written consent of UM having been first had and obtained;
(6) I am fully aware that if in the course of making this work I have infringed any copyright whether intentionally or otherwise, I may be subject to legal action or any other action as may be determined by UM.

Candidate's Signature **Date**

Subscribed and solemnly declared before,

Witness's Signature	**Witness's Signature**
Name:	**Name:**
Designation	**Designation**
Date	**Date**

ABSTRACT

Pseudomonas aeruginosa is one of the main causes of healthcare-associated infections among hospitalized patients. Healthcare-associated infections predominantly lead to pneumonia, urinary tract infections, as well as skin and soft-tissue infections. This organism is commonly multiresistant and leads to morbidity and mortality. In this study, the resistance mechanisms of 88 clinical *P. aeruginosa* isolates from the Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur, Malaysia were evaluated. The antibiotics resistance profiles of these isolates were determined and this was followed by evaluating the expression levels of *AmpC* cephalosporinase, the multidrug efflux pumps, the *OprD* outer membrane porin and the penicillin binding protein (*PBP2*, *PBP3*). Selected clinical isolates that were resistant to imipenem and meropenem were evaluated for metallo-β-lactamase (MBL) and extended spectrum β-lactamases (ESBL) production. The antimicrobial agents tested in this work were piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem, meropenem and colistin. These agents were selected as representatives of the primary antibiotic classes used to treat *P. aeruginosa* infections.

The clinical specimens of *P. aeruginosa* isolates were isolated from urine (53.4%), wound (21.6 %), sputum (5.7%), blood (5.7%) and in dwelling medical devices (13.6 %) as shown in Table 3.1a. Samples were collected from patients hospitalized in the surgical (31), medical (20), orthopedic (13), paediatric (7), neurosurgery (7), intensive care unit (ICU) (5), otorhinolaryngology (ENT) (3) and gynaecology wards (2). Of these isolates, 47 were from urine, 19 from wounds, 12 from in dwelling medical devices, 5 from blood, and 5 from sputum.

In the isolates tested. The highest resistance was observed for gentamicin (94.0%), ciprofloxacin and ceftazidime (92%), imipenem (74.0%), meropenem (78%), amikacin (66.0%), piperacillin/tazobactam (58.0%), aztreonam (56.0%) and colistin (7.0%), with the surgical department having the highest of numbers of isolates resistant for all antibiotics except colistin with urine and wound specimens being most resistant to the antibiotics except for colistin.

The gene expression analysis of 88 *P. aeruginosa* isolates showed overexpression of efflux pump genes for *MexY* (82.0%, from 2.0 to 1731.0 fold), *MexB* (73.0%, from 2.0 to 50.0 fold), *MexEF* (68.0%, from 2.9 to 371), *MexZ* (66.0%) and *MexCD* (48.0%, from 2.0 to 522), while for *AmpC* overexpression it was 65.0% (from 10.4 to 1806.0 fold). Down regulation were noted for *OprD* (97.0%, from 0.2 to 0.7 fold), *PBP2* (77.0%, from 0.1 to 0.7 fold), *PBP3* (84.0%, from 0.01 to 0.7 fold) and *OprM* (65%, from 0.2 to 0.7) as compared to those of *P. aeruginosa* ATCC 27853.

Among the resistant isolates overexpressed for the *MexB* and *MexY* efflux gene, the lowest mRNA expression was noted for isolates resistant to colistin, whereas the highest mRNA expression was noted for isolates resistant to ciprofloxacin and amikacin respectively. For the overexpression of *MexCD* and *MexEF* genes, the lowest mRNA expression was seen with isolates resistant to piperacillin/tazobactam. However, the highest overexpression for *MexCD* was seen with isolates resistant to meropenem and aztreonam while the highest overexpression for *MexEF* gene was noted with isolates resistant to colistin. For the overexpression of *AmpC* gene, the lowest mRNA expression was seen with isolates resistant to colistin and the highest mRNA expression was noted with isolates resistant to piperacillin/tazobactam. All isolates resistant to meropenem, imipenem and colistin demonstrated lower mRNA expression for *OprD* gene while as for *OprM* gene, the lowest

mRNA expression was observed in isolates resistant to amikacin. For the *PBP* gene, isolates resistant to colistin had lower mRNA expression for *PBP2*, *PBP3* while those resistant to imipenem had higher mRNA expression. With regard to the significance of the above results, the overexpressions of *MexY* gene were significantly different in isolates resistant to amikacin, gentamicin and ciprofloxacin as compared to other antibiotics ($p < 0.05$), while, for the *AmpC* gene, mRNA expression in *P. aeruginosa* isolates demonstrated high significant differences towards piperacillin/tazobactam antibiotics as compared to other antibiotics ($p < 0.05$).

Sixty-five of the clinical *P. aeruginosa* isolates that were resistant to imipenem and meropenem were then evaluated for detection of 6 different metallo-beta-lactamase (MBL) genes (bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM} and bla_{NDM}). In addition, these isolates were tested for the extended spectrum beta-lactamase (ESBL) production genes (bla_{VEB}, bla_{TEM}, bla_{CTX-M}, bla_{SHV} and bla_{PER}) using PCR. Among the 65 imipenem and meropenem resistant, 41 isolates were metallo-β-lactamase (MBL) producers and these genes bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{NDM} and bla_{SIM} were detected in 20, 14, 4, 2, and in 1 isolates respectively. Thirty-three of these 65 isolates tested positive for 3 ESBL genes out of which PCR positive isolates were present in 25 isolates for the bla_{VEB} gene, 5 isolates for the bla_{TEM} gene and 3 isolates for the bla_{CTX-M} gene respectively.

Most of the *P. aeruginosa* clinical isolates had a high level of resistance to examined antibiotics except colistin. Multidrug resistance phenotype in these clinical isolates was caused by the interaction of several different resistance mechanisms occurring within the same strain such as overexpression of efflux, *AmpC* overproduction or decreased outer membrane porin *OprD,* alteration of penicillin binding protein. Additionally, these strains highlight the ability of *P. aeruginosa* to develop dual resistance to different classes

of antimicrobial agents through independent mechanisms of resistance and highlight the need for the judicious use of therapy when dealing with *P. aeruginosa* to prevent multidrug resistance. The clinical *P. aeruginosa* isolates that were resistant to imipenem and meropenem demonstrated high efflux overproduction, MBL and ESBL production that confirm these resistance genes has the ability to increase the resistance to imipenem and meropenem among *P. aeruginosa*.

ACKNOWLEDGEMENT

I would like to thank my supervisors Associate Professor Dr. Mohd Yasim Mohd Yusof and Professor Dr. Shamala Devi for their constant supervisions, guidance, support and encouragement me throughout my study.

I would also like to thank all of my colleagues and my lab-mates in Medical Microbiology Department for their friendship. I would like thank Dr. Seok Muei and Dr. Mohammed Alhoot and Dr. Negar Shafiei Sabet for their assistance and advices. I would like to express my thanks to Dr. Hesham Mahyoub Sarhan Al-Mekhlafi from department of Parasitology, Faculty of Medicine and Dr. Ahmed Abdullah Madfa from Faculty of dentistry, University of Malaya for giving me constant support, valuable guidance and encouragement throughout my study. I wish to acknowledge UM, Faculty of Medicine and Institute of Postgraduate Studies (IPS) for providing me all the facilities for this research and funding (Research grants PPP No. PS184/2010A, Research grant /UMRG No.RG215/10 HTM and HIR F 000 009-21001).

Deepest thanks for mother, wife and brothers for their silent sacrifices, love and prayer during my study.

I gratefully acknowledge my brothers for the financial supporting during the course of this study.

TABLE OF CONTENTS PAGE

LIST OF FIGURES

LIST OF TABLES

LIST OF ABBREVIATIONS

%	Percentage
(ε)	Epsilon
<	Less-than
>	Greater-than
≤	Less-than or equal to
≥	Greater-than or equal to
°C	Degree celcius
µg	Microgram
µL	Microliter
A	Absorbance
AACs	Acetyltransferases
ABC	ATP-binding cassette
AIDS	Acquired immune deficiency syndrome
AMEs	Aminoglycoside modifying enzymes
ANTs	Nucleotidyltransferases
APHs	Aminoglycoside O-phosphotransferases
API	Analytical profile index
ATCC	American type culture collection
ATS	American Thoracic Society
$BaCl_2$	Barium chloride
bla	The structure gene of beta-lactamase
bp	Base pair
CAP	Community-acquired pneumonia
cDNA	Complementary DNA
CF	Cystic fibrosis
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CNS	Central nervous system
C_t	Crossing point
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
DNA	Deoxy ribonucleic acid
E	Efficiency
ESBLs	Extended spectrum β-lactamases
ExoS	Exoenzyme S
ExoT	Exoenzyme T
ExoU	Exoenzyme U
ExoY	Exoenzyme Y
g	Gram
G	Gram
GyrA	DNA gyrase mutation
H	Hour
H_2O	Water
H_2SO_4	Sulphuric acid
HAI	Healthcare-associated infections

I	Intermediate
ICU	Intensive care unit
IDSA	Infectious Disease Society of America
IL	Interleukin
ISS	intensive care unit surveillance study
kbp	Kilo base pairs
kDa	Kilodaltons
kg	Kilogram
L	Liter
LB	Luria Bertani
LPS	Endotoxin lipopolysaccharide
M	Molar
MATE	Multidrug and toxic compound extrusion
MBLs	Metallo-β-lactamases
MDR	Multidrug-resistant
MDRPA	Multi drug resistant *P. Aeruginosa*
MF	Major facilitator
MFP	Membrane fusion protein
MHA	Mueller-Hinton agar
MIC	Minimum inhibition concentration
min	Minute
mL	Mililiter
mm	Milli meter
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NaOH	Sodium hydroxide
NCBI	National Center for Biotechnology Information
nm	Nano meter
O_2	Oxygen
OD	Optical density
ORF	Open reading frame
OXA	Oxacillin
pABA	P-aminobenzoic acid
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
QRDR	Quinolone-resistant-determinative region
QS	Quorum-sensing
R	Resistant
RBS	Ribosome binding site
RNA	Ribonucleic acid
RND	Resistance-nodulation-cell division
rpm	Round per minute
RT-PCR	Reverse transcription polymerase chain reaction
S	Susceptible
sec	Second
SHV	Sulphydryl variable
SMR	Small multidrug resistance

spp.	Species
TBE	Tris-borate EDTA
TE	Tris-EDTA
TEM	Temoniera
Tm	Melting temperature
Trp	Tryptophan
Tyr	Tyrosine
UV	Ultraviolet
V	Volt
v/v	Volume/volume
VAP	Ventilator-associated pneumonia
w/v	Weight/volume
WHO	World health organization
Zn	Zinc

Chapter 1

Introduction

1.1 Introduction

P. aeruginosa is one of the main causes of healthcare-associated infections among hospitalized patients. Healthcare-associated infections predominantly lead to pneumonia, urinary tract infections as well as, skin and soft-tissue infections. This organism commonly demonstrates multiresistant isolates, which leads to morbidity and mortality (Aloush *et al.*, 2006; Giske *et al.*, 2008b; Park *et al.*, 2011). *P. aeruginosa* is a therapeutic challenge for the treatment of healthcare-associated infections. The choice of suitable antipseudomonal agents for treatment is significant in optimizing clinical effects (Micek *et al.*, 2005).

The extensive use of antimicrobial agents and the evolution of antimicrobial resistance strategies of bacteria have resulted in the emergence of nosocomial bacterial pathogens with acquired resistance to almost all available antimicrobial agents. These 'Superbugs', have severely threatened therapeutic choices in the last few decades (Livermore *et al.*, 2001). *P. aeruginosa* is considered multidrug resistant (MDR) if the isolate is resistant to more than two of the following drugs: piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem, meropenem and colistin (Mangoni *et al.*, 2008; Kiser *et al.*, 2010). These agents are representatives of the primary antibiotic classes used to treat *P. aeruginosa* infections. *P. aeruginosa* uses distinctive mechanisms to become resistant to a wide range of antimicrobials agents. Several mechanisms contribute to *P. aeruginosa* acquiring resistance. These include the up-regulation of efflux systems, decreased outer membrane permeability and β-lactamase production. However, acquired extended spectrum β-lactamase (ESBL) and Metallo β-lactamase (MBL) mediated resistance is important in emerging resistance mechanisms in *P. aeruginosa* (Dumas *et al.*, 2006; Strateva & Yordanov, 2009). *P. aeruginosa* has been reported to have constitutive expression of multidrug efflux pumps and *AmpC* β-lactamase, associated with loss in permeability of the outer membrane (De

Kievit *et al.*, 2001). A number of broadly specific efflux pump systems, including MexB-OprM and MexXY-OprM that belong to the resistance-nodulation-division family, supplies the main role to this intrinsic multidrug resistance. These systems are able to pump out multiple antipseudomonal compounds and reduce its susceptibility (Hocquet *et al.*, 2006).

Several studies on the MexAB-OprM mutants showed that this multidrug efflux pump extrudes aminoglycosides, macrolides, quinolones and most β-lactams but not imipenem (Xavier *et al.*, 2010). MexXY-OprM is however capable of extruding amikacin, ciprofloxacin, gentamicin, cefotaxime, meropenem, cefepime and erythromycin (Poole & Srikumar, 2001). *P. aeruginosa* also possesses inducible chromosome-encoded *AmpC* β–lactamase that belongs to the molecular class C that confers resistance to β-lactams (cephalosporins, penicillins). The up-regulation of efflux pumps coupled with overexpression of β-lactamase in addition to porin loss, may increase the pump rate and co-resistance phenotypes that could critically influence antimicrobial therapy in clinical settings (Poole, 2007; Quale *et al.*, 2006). In *Enterobacteriaceae* and *P. aeruginosa,* ESBLs are commonly known to mediate resistance to penicillin, cephalosporins, and monobactams (Tenover *et al.*, 2003). ESBL of Ambler class A enzymes are naturally able to hydrolyze 3rd generation cephalosporins and/or several carbapenems such as *CTX-M*, *TEM* ,*VEB*, *GES*, *SHV* and *PER* (Bonnet, 2004). The appearance of ESBL-producers associated with multiple resistant isolates pose a serious problem in the hospital setting. The factors that contribute to the increase in antibiotic resistance in bacterial pathogens are the extensive use of antibiotics coupled with the transmissibility of resistance determinants mediated by plasmids, transposons, and gene cassettes in integrons (Kang *et al.*, 2005).

The metallo β-lactamases (MBLs) class B is encoded by genes such as *IMP*, *VIM*, *SPM*, *GIM* and the recently reported *NDM*. The *IMP* and *VIM* types are the most common and widespread as they exhibit a worldwide distribution for which several allelic variants are known (Pagani *et al.*, 2005; Walsh *et al.*, 2005; Aggarwal *et al.*, 2008). The occurrence of ESBLs and MBL-producing *P. aeruginosa* are increasingly being reported worldwide (Gibb *et al.*, 2002; Pagani *et al.*, 2004; Lee *et al.*, 2005; Aggarwal *et al.*, 2008). The expression of the genes involved in the various mechanisms of the drug resistance were evaluated using the conventional method of detecting proteins by western blotting, which is complex and time consuming (Poole, 2007; Dumas *et al.*, 2006; Quale *et al.*, 2006; Hocquet *et al.*, 2006; Savli *et al.*, 2003). Current methods include real time PCR her quantification of mRNA expression of the target gene (Dumas *et al.*, 2006).

1.2 Characteristics of *P. aeruginosa*

P. aeruginosa is an aerobic Gram-negative rod (Figure 1.1), with the remarkable adaptable capacity to stay alive and continue under a wide range of environmental situations. This organism is usually 1.5-5 μm in length, 0.5-1.0 μm in width, and is motile due to the presence of flagella. *P. aeruginosa* was first isolated from a range of environmental resources by Schroeter in 1872 (Palleroni, 1984). In addition, this organism was first isolated from wound infections of soldiers whose bandages had a blue and green color by Carle Gessard in 1882 (Gessard, 1984). *P. aeruginosa* belongs to the *Pseudomonadaceae* family in the category of Gammaproteobacteria. Typical features of *P. aeruginosa* isolates include positive oxidase tests, the ability to be cultivated at temperatures of 42°C and (although it is classified as strictly aerobic) the ability to grow under anaerobic conditions by the presence of an alternative terminal electron acceptor such as nitrite or arginine, while utilizing more than eighty organic compounds as carbon and energy sources (Dworkin & Falkow, 2006).

Figure 1.1: Gram stain of *P. aeruginosa* cells (takes from http:// textbook of bacteriology.net/pseudomonas.html).

P. aeruginosa is equipped with an equally large repertoire of pathogenic mechanisms and is able to infect eukaryotic organisms ranging from amoeba to humans. *P. aeruginosa* is a glucose and lactose non-fermenting bacterium that is commonly isolated from hospitalized patients and may be found in a variety of aqueous solutions, including disinfectants, soaps and eye drops. It can also be found in sinks, hot tubs, respiratory equipment, showerheads, and has the ability to produce the water-soluble pigments pyoverdine and pyocyanin, which together confer the bright green color characteristic of the organism when grown on *Pseudomonas* isolation agar (King et al, 1954; Todar, 2006).

P. aeruginosa can cause a variety of diseases and can be isolated from almost any type of specimen. Infections can be acquired from the community or during a stay in a healthcare facility. *P. aeruginosa* is rarely part of the microbial flora of healthy individuals, but may colonize the gastrointestinal tract of hospitalized patients, particularly those who have received previous antibiotic therapy (Dworkin & Falkow, 2006). The colony morphology, pigmentation of *P. aeruginosa* can be quite substantially heterogeneous. On a simple agar culture at 37°C, the morphological colony

is smooth and large with an elevated center giving it the appearance of a fried egg. Colonies are usually pigmented and in actuality, the species designation, coming from the aeruginous meaning “the color of copper rust,” reflects the feature blue green color reported on colonies by the phenazine pyocyanin pigment. The rusty-brown phenazine pyorubin and fluorescent yellow-green siderophore pyoverdin are other common pigments, which are included in this bacterium (Palleroni, 1984). As pointed out by Lau *et al*. (2004) pyocyanin is a compound with a redox-active effect and has multiple cytopathic influences on mammalian cells (Lau *et al.*, 2004). *P. aeruginosa* has different forms of motility, which can be identified as swimming, twitching and swarming. Versatile, with motility chemotaxis that may allow it to move either in fluid and/ solid surfaces, *P. aeruginosa* swims in aqueous environments with single polar flagella movements. However, it can also move by twitching and swarming on solid surfaces. Flagella and type IV pili are needed for swarming whereas; twitching is powered by the sequential extension and retraction of type IV pili (Kohler *et al.*, 2000; Merz *et al.*, 2000). Many chemicals can be demonstrated to result in chemotactic responses due to the twitching and swimming of the *P. aeruguinosa* (Kato *et al.*, 2008).

The increasing use of the antimicrobial and environmental stresses leading to chronic infections of individual airways is due to change in colony morphology that arises during biofilm production (von Gotz *et al.*, 2004; Häußler *et al.*, 2003; Drenkard & Ausubel, 2002). According to von Gotz *et al*. (2004) and Boles *et al*. (2004) various types of colonial morphology in *P. aeruginosa* displays phenotypic modifications in motility, antibiotic resistance, biofilm formation, adherence properties, and virulence gene expression. The creation of various phenotypic properties may possibly contribute to the persistence and pathogenic achievement of *P. aeruginosa* (von Gotz *et al.*, 2004; Boles *et al.*, 2004).

1.3 Clinical infection caused by *P. aeruginosa*

P. aeruginosa can cause infection in almost any part of the body, although it does not typically cause infection in a healthy host. This bacterium is an opportunistic pathogen that causes respiratory system infections, urinary tract infections, dermatitis, bacteremia, soft tissue infections and a variety of systemic infections. It is found mainly in patients with severe burns, cancer, and AIDS patients who are immunocompromised. Outbreaks caused by this organism have been reported in diverse settings (Pirnay *et al.*, 2003). *P. aeruginosa* causes bacteremia mainly in immunocompromised patients and other diseases such as diabetes mellitus, hematologic malignancies, neutropenia and severe burns. The majority of *Pseudomonas* bacteremia is acquired in hospitals and nursing homes, which accounts for 25% of all healthcare-associated Gram-negative bacteremia. *P. aeruginosa* causes meningitis and brain abscesses due to the organism invading the central nervous system (CNS) from the paranasal sinus or inner ear, or if it is inoculated directly by means of surgery or invasive diagnostic procedures, or if it spreads from a distant site of infection such as the urinary tract. *P. aeruginosa* causes ear infection as a predominant bacterial pathogen in some cases of external otitis, including swimmer's ear. This bacterium is also found in the normal ear, but often inhabits the external auditory canal in association with inflammation, injury, maceration, or simply wet and humid conditions. *P. aeruginosa* is also a common cause of eye infections including keratitis, and has been isolated as the etiologic agent of neonatal ophthalmia (Martin *et al.*, 2003). *P. aeruginosa* also causes chronic contiguous osteomyelitis, usually resulting from direct inoculation of bone and is the most common pathogen implicated in osteochondritis after puncture wounds of the foot. *P. aeruginosa* can produce disease in any part of the gastrointestinal tract from the oropharynx to the rectum (Driscoll *et al.*, 2007).

The infection caused by *P. aeruginosa* varies depending on whether the person has an underlying disease, or has had some type of healthcare intervention (Weinstein, 1998).

As reported by System, (2003) *P. aeruginosa* is considered the most common cause of healthcare-associated pneumonia (17%), urinary tract infection (7%), surgical site infection (8%), blood pathogen (2%) and is the fifth most common isolate from all sites (9%) (System, 2003). *P. aeruginosa* is lately known as a common source of many community and healthcare-associated infections. In the community, the incidence of *P. aeruginosa* can cause community-acquired pneumonia (CAP) which is increased in patients with chronic obstructive pulmonary disease, nursing home residents and patients recently discharged from the hospital (Ruiz *et al.*, 1999). It is commonly spread in natural environments and is usually found in soil and water. It can grow in distilled water due to gaseous dissolved nutrients (Niederman, 2010; Lim *et al.*, 2003). Most aqueous solutions including disinfectants, antiseptics, intravenous fluids and eyewash solutions are considered as reservoirs of *P. aeruginosa*. It is common in hospital settings and persists in respiratory equipments, sinks, tubs and hydropathy baths. Because of their presence in soil they are frequently recovered from fresh vegetables and plants (Weinstein & Hota, 2004).

The infections caused by *P. aeruginosa* range from self-limiting folliculitis to life-threatening bacteremia. These infections occupy the cornea, respiratory tract, urinary tract, blood stream, surgical sites and skin infections in the setting of burn injuries, even though infections may occur effectively in every anatomical site. It also leads to urinary tract infections and ventilator-associated pneumonia in the intensive care unit (Speert *et al.*, 2002; Mittal *et al.*, 2009). The infections resulted by *P. aeruginosa* can be divided into acute or chronic. It has been quoted that acute infections, such as ventilator-associated pneumonia, are invasive, cytotoxic and commonly lead to systemic infection,

septic shock and mortality (Martin & Yost, 2011; Agodi *et al.*, 2007; Martínez-Solano *et al.*, 2008). On the contrary, the chronic respiratory infections linked with cystic fibrosis, although heavy in colonization of the sputum (>10^8 colony-forming units cfu/g), are non-invasive, noncytotoxic and infrequently develop to systemic infection. These chronic infections may continue for decades and in the end lead to lung deterioration and mortality (Speert *et al.*, 2002; Scheetz *et al.*, 2009).

P. aeruginosa colonization plays a main role within intensive care units (ICUs), and it has the capability to colonize in-patients, with ICUs clearly established as endemic settings (Erbay *et al.*, 2003). A deep understanding and knowledge of the mechanisms of *P. aeruginosa* is therefore critical. Risk factors considerably related with the acquisition of this bacterium in ICUs include: period of stay, mechanical ventilation (Talon *et al.*, 1998), long-term use of antibiotics (Blanc *et al.*, 1998; Baddour *et al.*, 1995; Carmeli *et al.*, 1999a), alcoholism (Blanc *et al.*, 1998; Talon *et al.*, 1998) and the utilization of indwelling urinary catheters (Blanc *et al.*, 1998). *P. aeruginosa* is infrequently found as a part of the human microflora of healthy individuals as this organism dies in the dry skin of healthy individuals (Bellais *et al.,* 1999). *P. aeruginosa* has a vast array of virulence factors and is universally distributed in natural environments. This organism is rarely responsible for community-acquired infections in healthy individuals. On the other hand, the incidence of *P. aeruginosa* associated infection is high in a hospital environment particularly in immune compromised individuals, epithelium compromised cystic fibrosis patients, and individuals with severe burns, ulcerations and mechanical abrasions caused by catheterization. *P. aeruginosa* is the main cause of death in cystic fibrosis patients (Thuong *et al.*, 2003). Infections caused by *P. aeruginosa* not only become increasingly resistant to one drug, as multi-drug resistance is also increasing and have been difficult to treat (Flamm *et al.*, 2004). Due to increasing rates of antibiotic resistance in *P. aeruginosa,* the treatment of

infections is still a serious medical challenge. Infection with MDR *P. aeruginosa* is associated with risk factors including severity of illness, invasive devices, a bedridden state, and in hospitalized patients, lead to increased length of stay and increased therapeutic costs as well as significant morbidity and mortality (Defez *et al.*, 2004; Paramythiotou *et al.*, 2004; Moore & Flaws, 2011b). *P. aeruginosa* has been a well known cause of infections for almost 130 years and will probably remain a leading cause of infections in humans for many years to come for various reasons (Moore & Flaws, 2011c); *P. aeruginosa* is ubiquitous in nature, increasing the probability of exposure, patients with chronic disease and immunosuppression for long periods are susceptible hosts for infection with *P. aeruginosa*. In addition, *P. aeruginosa* has many resistant mechanisms to antipseudomonal agents that are not always used judiciously and when overprescribed drive the development of resistance.

1.4 Pathogenicity of *P. aeruginosa*

P. aeruginosa is an opportunistic pathogen that causes acute and chronic infections that can be acquired from the environment. It colonizes the respiratory epithelium in patients with predisposing conditions such as cystic fibrosis, mechanical ventilation, immunodeficiency or preexisting respiratory disease. The *P. aeruginosa* pathogenicity is mainly caused by various bacterial virulence factors and genetic flexibility, enabling it to survive in varied environments. Lung injury associated with *P. aeruginosa* infections result from both the direct destructive effects of the organism on the lung parenchyma, and the exuberant host immune responses. The factors that contribute to the pathogenesis of *P. aeruginosa* are based on the health status of the host. Hospitalized patients with underlying disease, particularly those who are on a ventilator are most at risk for pneumonia caused by *P. aeruginosa*. In addition, pathogenesis comes from the organism itself. In order for *P. aeruginosa* to cause any type of infection it must first enter the host and colonize. Entry is often through inhalation into

the respiratory tract, but the organism is so ubiquitous that is hard to tell exactly how the organism is acquired in all cases.

The virulence factors produced by *P. aeruginosa* are listed and summarized in Table 1.1 (Sadikot *et al.*, 2005; Driscoll *et al.*, 2007). All of the virulence factors used by *P. aeruginosa* are also produced by other microorganisms except for pyocyanin, which is uniquely produced by *P. aeruginosa*. Many of these factors assist colonization, whereas others facilitate bacterial invasion. Several factors are required in bacterial colonization, including, pili or fimbriae, flagella and surface polysaccharides. Virulence factors can be divided into two functional groups: factors that assist in the attachment of the organism to host cells, which are the fimbriae and flagella; and factors that support in the invasion of tissue and the inhibition of the immune response. These virulence factors include pili, and flagella, which play an initial role in motility and adhesion to the epithelium, as well as the endotoxin lipopolysaccharide (LPS). These factors then perform a main function in the irreversible adhesion to epithelial cells, which is the first significant step in colonization of the respiratory epithelium. Upon cell contact, the type III secretion system, a major virulence determinant is activated. The type III secretion system permits *P. aeruginosa* to produce toxins into the host cell. The type III secretion system is coupled with acute invasive infections and requires pilin-mediated bacterial-epithelial contact (Feldman *et al.*, 1998; Hauser *et al.*, 1998). This system is activated on contact with eukaryotic cell membranes and interferes with signal transduction, leading to cell death or changes in host immune responses. The type III secretion system includes three apparatus: the secretion, the translocation or targeting apparatus, and the secreted toxins (effector proteins) and cognate chaperones (Gauthier *et al.*, 2003).

P. aeruginosa produces a number of secreted toxins by the single type III secretion system, which include exoenzyme S (ExoS), exoenzyme U (ExoU), exoenzyme T

(ExoT), and exoenzyme Y (ExoY). They contribute at differing levels in the cytotoxicity leading to invasion and dissemination of bacterium (Epelman *et al.*, 2004). Among these exoenzymes, ExoU may be responsible for the highest virulence (Hauser *et al.*, 2002; Schulert *et al.*, 2003). The expression of the type III secretion system in *P. aeruginosa* isolates has been associated with increased mortality in patients with pneumonia, sepsis, respiratory failure, and more severe diseases (Hauser *et al.*, 2002; Roy-Burman *et al.*, 2001). Aside from the type III secreted proteins; *P. aeruginosa* produces additional virulence factors that contribute to its pathogenicity. These virulence factors are produced by the type II secretion system into the extracellular space. These include exotoxin A, alkaline phosphatase, elastase and phospholipase C, which contribute in the invasion via destroying the protective glycocalix of the respiratory epithelium and exposing epithelial ligands to *P. aeruginosa* (Lau *et al.*, 2005; Hauser *et al.*, 2002). These proteins encourage invasion by causing cytotoxic effects on host cells (Wilson *et al.*, 1987). A similar function also exists for pyoverdine and pyocyanin.

P. aeruginosa strains mainly produce pyocyanin (N-methyl1-hydroxyphenazine), the pigment that gives the blue-green color to the bacterial colonies. It has been found to have various pathogenic effects such as increasing production IL-8, depressing host-response (Denning *et al.*, 1998; Leidal *et al.*, 2001) and inducing apoptosis in neutrophils (Allen *et al.*, 2005). In animal models of acute and chronic lung infection, pyocyanin was shown to be necessary to *P. aeruginosa* virulence (Lau *et al.*, 2004). In acute infections, invasion, dissemination and extensive tissue damage predominate. In chronic infections, primarily in cystic fibrosis patients, *P. aeruginosa* may also adjust, by losing its most immunogenic features such as flagella and pili to prevent clearance, and by isolating itself from host defenses and adhering to the respiratory epithelium by forming biofilms. A persistent inflammatory state is maintained by extracellular

secreted virulence factors (Landsperger *et al.*, 1994). Whether in acute or chronic infections, *P. aeruginosa* possesses a multiplicity of regulatory systems allowing it to adapt to its environment and notably to host defenses (Mahenthiralingam *et al.*, 1994). Among these systems, quorum sensing (QS) displays *P. aeruginosa* adaptability (Finch *et al.*,1998). Quorum sensing systems are complex bacterial cell-to-cell signaling systems that allow the bacteria to sense their own cell density and to communicate with each other resulting in coordinated production of virulence factors depending on bacterial density (Kaufmann *et al.*, 2008). QS has been shown to be critical in maintaining airway inflammation through virulence factor production and to the formation of biofilm in chronic infections (Hentzer *et al.*, 2003).

Table 1.1: Virulence factors of *P. aeruginosa* and their function

Virulence Factor	Function
Fimbriae	Attachment to host cells and activation of pro inflammatory gene expression
Polar flagella	Motility, attachment to host cells and activation of lnterleukin-8
Type III secretion system	Injects toxins (ExoS, ExoT, ExoU, ExoY) into host cells
ExoS	Stimulates tumor necrosis factor alpha production
ExoT	Activates GTPase
ExoU	Cytotoxin
ExoY	Adenylate cyclase activity
Quorum-sensing molecules	Coordinate expression of genes among other pseudomonal cells and promotes the formation of biofilms
Pyochelin and pyoverdin	Bind iron
Elastase, proteases, hemolysins, and leukocidin	Aid in tissue invasion and lyse host cells
Pyocyanin	Inhibits lymphocyte proliferation and cilia function and produces reactive oxygen intermediates
Exotoxin A	Inhibits protein synthesis in host cells and helps organism disseminate
Lipopolysaccharide	Endotoxin
Alginate	Free radical scavenger; inhibits phagocytosis, neutrophils chemotaxis and activation of complement

1.5 Treatment of *P. aeruginosa*

P. aeruginosa is often resistant to a wide range of antimicrobial agents, including fluoroquinolones, tetracycline, aminoglycoside, β-lactams, macrolides, rifampicin, cotrimoxazole (trimethoprim/sulfamethoxazole) and chloramphenicol. Several indigenous resistance mechanisms elucidate this pattern, such as active efflux pumps, low membrane permeability and production of β-lactamases (Hancock, 1998). *In vitro* susceptibility data are essential guides for the choice of a proper antibiotic for *P. aeruginosa* infections, due to the incidence and diverse acquired resistance found by clinical isolates. Antimicrobials that are typically considered effective toward *P. aeruginosa* can be generally classified into the following seven groups:

First are the penicillins, such as ticarcillin and piperacillin, β-lactam/β-lactamase inhibitor combinations including ticarcillin/clavulanic acid, and piperacillin/tazobactam (Lister, 2000). Lodise *et al*. (2007) showed that extended infusion of piperacillin/tazobactam (4 hours vs 1 hour) might target pharmacodynamic targets more proficiently in severely ill patients (Lodise *et al.*, 2007). In contrast, the Clinical Laboratory Standards Institute resistance breakpoint for piperacillin/tazobactam suspicions have expressed concern that suitability of this drug (Tam *et al.*, 2008) may lead to therapeutic failures due to the false-susceptibility results.

Second are the cephalosporins, which include ceftazidime, cefoperazone and cefepime (Bush, 2002). From all third generation cephalosporins, ceftazidime is the only one active against *P. aeruginosa.* Among fourth generation cephalosporins, cefepime is the only one that is accepted in the United States for utilization in human beings and has an extended range. Cefepime is active toward Gram-positive and Gram-negative bacteria, including *P. aeruginosa*. The fourth generation cephalosporins exhibit chemical properties that can result in increased resistance by Gram-negative microorganisms (Zuanazzi *et al.*, 2010).

Third are the monobactams, this class of drugs is only represented by aztreonam (Jones *et al.*, 2002). Aztreonam is mainly used against gram-negative aerobic microorganisms, as well as *P. aeruginosa* and *Klebsiella*. It is also recommended for use in urinary tract infections, soft-tissue infections, intra-abdominal and pelvic infections and pneumonia (Ennis & Cobbs, 1995).

Fourth are the carbapenems, which include ertapenem, doripenem, imipenem and meropenem. Meropenem is fast becoming more effective than imipenem with regard to resistance (Livermore, 2001b). Extended infusions of meropenem (3 hours) have been found to results in positive outcome (Santos Filho *et al.*, 2007). *In vitro* data proposes a two-fold advantage for doripenem above meropenem, a four-fold advantage to imipenem (Jones *et al.*, 2004b) and decreased possibility for resistance selection (Mushtaq *et al.*, 2004). In addition, sustained infusions may increase the antipseudomonal efficacy of doripenem in infections sourced by less susceptible strains (Bhavnani *et al.*, 2005).

Fifth are the fluoroquinolones, levofloxacin and ciprofloxacin. As judged by MIC, ciprofloxacin is more potent than levofloxacin. However, maximum doses of levofloxacin have superior pharmacokinetics, which lead to balance the decreased potency. Therefore, these two fluoroquinolones are pharmacodynamically effective against *P. aeruginosa* (Tennenberg *et al.*, 2006).

Sixth are the aminoglycosides, which include amikacin, tobramycin, gentamicin, and netilmicin. In their *in vitro* study, Giamarellou *et al.* (1984) found that aminoglycosides when combined with β-lactams has a synergistic role (Giamarellou *et al.*, 1984), particularly, with amikacin combinations as stated by Giamarellos-Bourboulis *et al.* (2005) (Giamarellos-Bourboulis *et al.*, 2005).

Finally the polymyxin (colistin), which has lately re-appeared as an effective solution to treat MDR *P. aeruginosa* (Giamarellou & Kanellakopoulou, 2008; Falagas *et al.*, 2005c; Michalopoulos & Falagas, 2008; Kasiakou *et al.*, 2005; Falagas *et al.*, 2005b). The toxicity of this drug (nephrotoxicity, but also neuromuscular blockade and ototoxicity) has been much discussed but has been reported to be controllable and lower than initially thought (Falagas *et al.*, 2005b). Primary experimental data recommends that colistin can be managed even in an aerosolized form to mechanically ventilated, non-cystic fibrosis patients with pseudomonal infections (Horianopoulou *et al.*,2005). Currently, colistin is becoming the foremost curative choice for strains with complicated MDR patterns (Falagas *et al.*, 2006a; Kallel *et al.*, 2007; Hachem *et al.*, 2007). Some confusion still exists concerning the different formulations used (Falagas & Kasiakou, 2006). Polymyxin B belongs to this group, while clinical knowledge on this drug is usually limited. It has been reported that Polymyxin B has been used as a rescue therapy in a series of patients with healthcare-associated pneumonia caused by MDR *P. aeruginosa* (Furtado *et al.*, 2007). As a part of these antimicrobial classes, rifampicin has been suggested to be used in combination with this agent due to the potential synergistic results observed (Giamarellos-Bourboulis *et al.*, 2003). Fosfomycin is an additional agent that *in vitro* shows some efficacy for use in pseudomonal infections, while relevant research has been limited (Falagas *et al.*, 2010; Falagas *et al.*, 2009).

P. aeruginosa is considered a serious therapeutic challenge for the treatment of both health care and community associated infections. To optimize the clinical outcome the choice of a suitable antipseudomonal drugs at the start of therapy is important (Bisbe *et al.*, 1988; Micek *et al.*, 2005). Unfortunately, the choice of the most proper antipseudomonal is difficult due to the capability of *P. aeruginosa* to develop resistance to various groups of antipseudomonal drugs, even through the course of treating an infection. Epidemiological reports have found that the rise in morbidity and mortality

are associated with infections caused by drug-resistant *P. aeruginosa*. The early treatment is significant to the outcome of the infection and adequate empirical regimens for *P. aeruginosa* treatment should be started before receipt of the results of cultures and susceptibility testing. Numerous aspects should be considered in the case of selection. These include the empirical treatment, including the source and nature (community-acquired vs. healthcare-associated infections) of the infection, diseases, underlying risk factors (e.g., length of hospitalization, ICU admissions, previous antimicrobial chemotherapy), data about the epidemiology of resistance phenotypes in the individual setting, hospital prescription policies, pharmacokinetic parameters and overall cost of treating the infection (Aloush *et al.*, 2006; Carmeli *et al.*, 1999b; Gasink *et al.*, 2006).

The drug resistance development leads to limited therapeutic choices for treatment of severe infections. In recent years, infections caused by MDR *P. aeruginosa* have become a serious problem, especially in the healthcare-associated settings (Bassetti *et al.*, 2011). The World Health Organization (WHO) has identified antimicrobial resistance as one of the three most important problems to human health (Rice, 2008), and as a consequence the theme of World Health Day, on April 7, 2011, was "Antimicrobial Resistance: no action today and no cure tomorrow" (Piddock, 2011). Resistance to the current library of antimicrobial agents is a serious problem worldwide including Europe, Latin America, North America and the Asia-Pacific region (Dupont *et al.*, 2001; Kollef, 2006; Livermore, 2012). The use of common antipseudomonal agents that are mentioned above in the treatment of *P. aeruginosa* infections is a challenging task for clinicians. This is because most studies show significant differences in their efficacy and increased rates of resistance for these agents that lead to therapeutic failure (Surveillance, 2004; Lepper *et al.*, 2002; Driscoll *et al.*, 2007; Obritsch *et al.*, 2005; Moore & Flaws, 2011c; Dupont *et al.*, 2001; Kollef, 2006; Livermore, 2012).

Consequently, clinicians are forced to rediscover old drugs, such as polymixins and fosfomycin (Bassetti *et al.*, 2011). The antibiotic monotherapy for urinary tract infections caused by *P. aeruginosa* is usually recommended. However, it is not advised for the treatment of upper tract infections complicated by abscess formation, or whenever there is a suspicion of bacteraemia, or for infections in neutropenic patients.

In contrast, a combination chemotherapy including at least two different anti-pseudomonal agents is usually recommended for the treatment of healthcare-associated pneumonia infection, endocarditis, bacteraemia and severe *P. aeruginosa* infections (Young *et al.*, 2000). The rationale for combination chemotherapy is essentially to reduce the chances of selection of resistant mutants during therapy, as well as to exploit the potential synergistic activity of some agents. The preferred combination is still the aminoglycosides and β-lactams, as synergism between these drugs has been demonstrated by *in vitro* studies (Giamarellou *et al.*, 1984; Giamarellos-Bourboulis *et al.*, 1996; Burgess & Hastings, 2000). Controversy revolves around the issue as to whether combination or monotherapy is more appropriate in the treatment of *P. aeruginosa* infections. There have been several retrospective studies and Meta analyses published in recent years in favour of both sides. One multicenter, retrospective study conducted by Garnacho-Montero and colleagues in 2007 sought to evaluate whether one antibiotic achieved equal outcomes compared to combination therapy in patients with ventilator-associated pneumonia (VAP) caused by *P. aeruginosa* (Garnacho-Montero *et al.*, 2007). They found that more patients on monotherapy were inappropriately treated than those who were on combination therapy. In addition, the mortality rate was higher amongst patients treated with only one drug. They concluded that combination therapy be used at least initially in order to increase the chances that at least one drug is effective before the organism is identification and susceptibility are known. According to consensus guidelines suggested by the Infectious Disease Society of America (IDSA)

and the American Thoracic Society (ATS), for empiric management of community or hospital-acquired pneumonia, the following combination is recommended in patients who have risk factors for *P. aeruginosa*: Use of an antipseudomonal quinolone plus an anti-pseudomonal beta-lactam, an aminoglycoside plus an anti-pseudomonal beta-lactam (the most common combination reported in the literature) and an aminoglycoside plus an anti-pseudomonal quinolone.

Selection of antibiotics should include consideration of local antimicrobic resistance patterns as well as institutional antibiograms. The consensus suggests a recommended therapy of two weeks, but state that the duration of therapy should be adapted to the patient's signs and symptoms of clinical improvement (Mandell *et al.*, 2007). The main reason behind the combined empirical therapeutic course is not the potential for synergy, but the improved opportunity of covering the responsible strain. For instance, in the case of monotherapy by using a quinolone as, for an average 80% susceptibility, there is a 1: 5 ability of a resistant strain and, therefore, therapeutic failure. When administering a combination dosage, the chances of curative failure are decreased because MDR is not as frequent as isolated resistance (Fish *et al.*, 1995). Most studies favor combination therapy for first treatment and management of *P. aeruginosa* infections. Those requiring an antimicrobial β-lactam and either fluoroquinolone or aminoglycoside, have long been considered to comprise a proper antipseudomonal treatment for infection caused by *P. aeruginosa* (Siegman-Igra *et al.*, 1998; Giamarellou, 2002; Kanj & Kanafani, 2011). Combination therapeutic regimens have many advantages: it is economically safer, has rapid bacterial killing capabilities and has lower pressure for selection of resistance (Amari *et al.*, 2001; Safdar *et al.*, 2004). In addition, combining two drugs *in vitro,* enhances clinical efficacy and prevents the emergence of resistant strains. The combination therapy is beneficial to the patient because of the potential for synergy, having the increased chance that at least one agent

will be effective, and the reduction of the emergence of resistant strains. The disadvantages of the combination therapeutic regimens are: increased frequency of adverse events (mainly as a result of the aminoglycoside component), increased risk for fungal super-infections, and increased cost (Paul *et al.*, 2004). However, there are benefits associated with monotherapy that should not be dismissed. These include lower health care costs and fewer adverse events especially when aminoglycosides are not administered. There is also the utilization of a narrower spectrum of treatment and the possibility of reducing the probability the patient could develop an infection with a multidrug resistant strain of *P. aeruginosa*.

A meta-analysis conducted by Safdar *et al*. (2004), found a mortality benefit of combination therapy for *P. aeruginosa* infection, which included activity in lung and abscesses and improved safety profile (Safdar *et al.*, 2004). The analysis recommended that a combination therapy comprising of a β-lactam with a quinolone might be better compared to a combination with aminoglycosides. This observation is supported by an *in vitro* pharmacokinetic mode (Lister & Wolter, 2005). Besides the effects of combination therapy mentioned above, an additional important feature of dual therapy is the widening of the empirical coverage. An MDR phenotype can be expected in special conditions such as in the case of a patient with VAP and a history of previous antibiotic administration, particularly carbapenems that may have chosen resistance. In this case, it allows for the empirical therapy use of colistin (Rios *et al.*, 2007; Falagas *et al.*, 2006b). To reduce the incidence of *P. aeruginosa* infection, the identification of patients at risk for infection and prevent exposure to the organism would be needed as well as to prevent the further development of resistance by analyzing resistance trends and adjusting therapeutic regimens regularly (Falagas *et al.*, 2006b). Studies have shown that antimicrobial pressure leads to the development of resistance, and in the absence of that pressure, the organism reverts to being susceptible. The assess resistance should be

continually needed, recognize trends and implement change as necessary. Only then could be come close to winning the war against such a powerful adversary (Moore & Flaws, 2011c). In the last decade, there have been no new antibacterial agents, effective toward MDR strains of *P. aeruginosa* that have been introduced (Rossolini & Mantengoli, 2005; Boucher *et al.*, 2009). The Infectious Diseases Society of America announced their "Bad Bugs No Drugs-10 by 20" initiative to support the development of ten new antibiotics by 2020 (Spellberg *et al.*, 2011; Boucher *et al.*, 2009). This initiative supports strengthening current approaches to minimize antimicrobial resistance, protecting effectiveness of the drugs currently available by maximizing hospital infection control practices, and limiting the spread of resistance (Kuehn, 2011).

1.6 Emergence of antibiotics resistance in *P. aeruginosa*

Hughes & Datta (1983) found low rate antibiotic resistance when screening *Enterobacteriaceae* strains collected in different parts of the world from 1917 to 1954. When a new antibiotic is established, a lot of bacteria are initially susceptible, however resistance development is often observed after a short time (Hughes & Datta, 1983). In the 1960s, *P. aeruginosa* emerged as a main human pathogen owing to its use for wound and burn victims, in addition to neutropenic, cystic fibrosis and surgical patients (Doggett & Aduan, 1979). In spite of the activity of anti-pseudomonal agents being one of the holy grails of pharmaceutical drug discovery for many decades, it remains one of the main recalcitrant and difficult to treat organisms. As therapeutic options run out, *P. aeruginosa* has attained "superbug" status. Antimicrobial resistance, which has become widespread today, seems to have been rare in the pre-antibiotic era (Moore & Flaws, 2011a). Antibiotic resistance among *P. aeruginosa* infection is an increasing global problem and a public health threat, posing many therapeutic challenges. Additionally, the susceptible strains of *P. aeruginosa* can acquire drug resistance during treatment,

commonly with a relatively high frequency (Quinn *et al.*,1986; Lolans *et al.*,2008; Mesaros *et al.*, 2007). The emergence of resistance in *P. aeruginosa* has been reported with all classes of antimicrobial agents including penicillins, cephalosporins, carbapenems, aminoglycosides and fluoroquinolones (Livermore, 2002; Lister *et al.*, 2009).

This emergence of resistance has limited the opportunity of therapeutic options and is leading to increased rates of morbidity and mortality and increased costs of treating patients with *P. aeruginosa* infection (Livermore, 2002; Moore & Flaws, 2011a). *P. aeruginosa* is considered the third most common healthcare-associated infections in our society giving rise to a wide range of opportunistic infections. Its high intrinsic resistance to antibiotics and ability to develop multidrug resistance poses serious therapeutic problems (Zuanazzi *et al.*,2010; Cao *et al.*, 2004a). The resistance of antimicrobial agents is constantly increasing with geographical differences frequently linked to local antibacterial strategies. The emergence of drug-resistance occurs frequently in the ICU and raised resistance is mainly a dilemma in ICUs, particularly in Europe (Jones *et al.*, 2004a). Generally, the fluoroquinolones are the antimicrobial class where the resistance rates are rising quickly (though, geographical difference subsists) with resistance to β-lactams and aminoglycosides. In Europe, this is more important, even though the emergences of colistin-resistant strains are being reported (Matthaiou *et al.*, 2008; Walkty *et al.*, 2008; Denton *et al.*, 2002). A review (Bonomo & Szabo, 2006) described MDR as the decreased susceptibility to more than one of the following antimicrobial agents: quinolones, carbapenems, cephalosporins, β-lactam inhibitor combinations and aminoglycosides. Based on this description, a strain resistant to amikacin and ceftazidime will be considered MDR, while there are sufficient treatment options. Multidrug-resistant strains were isolated more frequently from ICU and nursing home patients. Manian *et al*. (1996) analyzed resistance rates among *P. aeruginosa*

isolated from an ICU and found that the rates of resistance to penicillins and cephalosporins was statistically related to prior treatment with third generation cephalosporins (Manian *et al.*, 1996). Overall, MDR rates for Europe are 5%, Asia and the US are 2%, while MDR rates for Latin America is more frequent at 8% of total isolates. When ICU isolates only are assessed, the pattern is diverse; in US resistance frequencies in ICU isolates are 20%, and in Asian ICUs they are 30%. There have also been growing numbers of reports of occurrences of widely resistant isolates (Rossolini & Mantengoli, 2005).

Numerous reports have found that multidrug-resistant strains of *P. aeruginosa* usually arise after prolonged exposure to antipseudomonal therapies (Georges *et al.*, 2006; Nadeem Sajjad Raja, 2007; Livermore, 2012) or after empirical treatment (Zavascki *et al.*, 2006; Arancibia *et al.*, 2002; Mesaros *et al.*, 2007). A number of *P. aeruginosa* strains which showed resistance to beta-lactams, reflect a previous treatment history of the patients with wide-range beta-lactams. For example, a considerable association was observed in a single medical center between the antecedent use of ceftriaxone, piperacillin, ceftazidime and cefotaxime. There was resistance to these drugs among *P. aeruginosa* strains that usually produce chromosomal *AmpC* beta-lactamase, including 155 isolates of *P. aeruginosa* (Lolans *et al.*, 2008). A study of the occurrence of *P. aeruginosa* resistance to β-lactam antibiotics in ICU patients found a high risk emerging with imipenem, piperacillin/tazobactam and cefotaxime during treatment (Georges *et al.*, 2006). A previous randomized assessment comparing imipenem to ciprofloxacin for the treatment of healthcare-associated pneumonia also illustrated a higher risk of emergence of resistance in the carbapenem arm (Aarts *et al.*, 2008). Several factors may lead to the increase in antibiotic resistance, such as hospital stay and period of hospitalization before ICU admission, surgical ICU stay, the type of operation, length of ICU stay, previous antibiotic use, and inappropriate use of antipseudomonal drugs and

inadequate adherence to infection control practices (Fridkin *et al.*, 2002; Neuhauser *et al.*, 2003; Siegel *et al.*, 2007). MDR has been associated with a two-fold prolongation of duration of hospitalization, a nine-fold elevation in secondary bacteraemia, a three-fold raise in mortality and major increases in healthcare costs (Carmeli *et al.*, 1999b). Significant data from ICU isolates of *P. aeruginosa* resulting from Europe are even worse. From 1990 to 1999 resistance to ceftazidime reached 57%, resistance to aminoglycosides reached 37-70%, resistance to imipenem reached 52%, resistance to ciprofloxacin reached 56% and resistance to piperacillin/tazobactam reached 53% (Rossolini & Mantengoli, 2005). The prevalence of MDR *P. aeruginosa* in the US was recently reported to have increased from 1% to 16% (D'Agata,2004). A multi-fold increase was noted by Jung et al. (Jung *et al.*, 2004) which reported 22% of *P. aeruginosa* isolates were resistant to any antipseudomonal agent in 1998, and 32% of isolates were resistant to at least three agents by 2002. In 2004 from the US National Nosocomial Infection Surveillance System (NNIS) published a report, which demonstrated the resistance rates with *P. aeruginosa* isolates to quinolones at 29.5% and imipenem at 21.1%. In ICU isolates, the particular rates of resistance were even higher- up to 23.6% for ceftazidime, 31.4% for piperacillin/tazobactam, 38% for imipenem and 51.6% for ciprofloxacin (Cardo *et al.*, 2004). Other studies in the intensive care unit surveillance study (ISS) database exhibited that *P. aeruginosa*, over a ten year period was becoming increasingly more resistant to antimicrobial agents (Georges *et al.*, 2006; Obritsch *et al.*, 2004; Kallel *et al.*, 2008).

1.7 Mechanisms of the antibiotic resistance in *P. aeruginosa*

Resistance of *P. aeruginosa* to antimicrobial agents arises through a variety of mechanisms, such as intrinsic resistance to antibiotics by a large selection of genetically encoded resistance mechanisms, acquisition of additional resistance mechanisms from other bacterial organisms in the form of plasmid-encoded genes and mutations in chromosomal genes (Tenover, 2006). *P. aeruginosa* is intrinsically resistant to certain antipseudomonal agents via three different mechanisms including antibiotics cleavage by β-lactamase enzymes, antibiotic ejection by chromosomally encoded efflux mechanisms and decreased drug uptake due to loss of outer membrane porin protein (Poole, 2004b; Pfeifer *et al.*, 2010). It has been observed that *P. aeruginosa* acquires resistance to antipseudomonal agents either by mutations to chromosomal genes or via the acquisition of plasmids encoding genes contributing resistance. Firstly, mutation of the *gyrA* gene alters the structure of DNA gyrase, which is the target of the quinolones (Lambert, 2002). When mutations occur in the respective genes, certain penicillin-binding proteins (PBPs) confer resistance to beta-lactams (Lambert, 2002). PBPs are important in the last steps of the synthesis of peptidoglycan, which is the most important constituent of bacterial cell walls. The name PBP is derived from their affinity for the binding of penicillin. PBPs catalyze a number of reactions required in peptidoglycan cross-linking from lipid intermediates and also the elimination of terminal D-alanine from the precursor of peptidoglycan (Spratt, 1977). Few studies have concentrated on the recognized role of alterations of penicillin-binding proteins in relation to the carbapenem resistance in Gram-negative bacteria (Ayala *et al.*, 2005; Giske *et al.*, 2008a).

Bellido *et al*. (1990) depicted a case of treatment failure, where the *P. aeruginosa* resistant isolates exhibited diminished binding of radioactively labeled imipenem to *PBP-4*. In addition, the authors detected that, following several routes in an antibiotic-

free broth, the isolate regained its capability to bind imipenem, and the MIC was reduced from 32 to 8 mg/L (Bellido *et al.*, 1990). Almost no follow-up studies have been carried out on the *P. aeruginosa* clinical isolates, and it is still unclear whether *PBP* alters in significant mechanisms of carbapenem resistance. One study has been done on other Gram-negative bacilli, as well as on the clinical isolates of *Acinetobacter baumannii*, which showed down-regulation of *PBP-2* associating well with reduced susceptibility to the carbapenems (Fernández-Cuenca *et al.*, 2003). Neuwrith *et al*. (1995) found that an imipenem-resistant *Proteus mirabilis* isolate would carry *PBP-2* with reduced affinity to imipenem (Neuwirth *et al.*, 1995). Besides this, a *PBP-2* mutant was found in *E. coli,* with increased imipenem MIC, however meropenem MIC was not affected, and this suggested that meropenem also binds to PBP-3 (Sumita & Fukasawa, 1995). In addition, Ayala *et al*. (2005) demonstrated that alterations of *PBP-3* might possibly reduce susceptibility to imipenem in *Bacteroides fragilis* (Ayala *et al.*, 2005). Alterations of *PBP-2* and *PBP-3* can be speculated as a carbapenem resistance mechanism in *P. aeruginosa.* Theoretically, down-regulation and the existence of amino acid changes occur in the area of the three conserved motifs S-X-N, K-T-G and S-X-X-K as shown in Figure 1.2 (Ghuysen, 1994). Mutations in active sites of *PBP-2* and *PBP-3* can be essential for carbapenem resistance, and more studies are required to concentrate on this issue. Mutations in genes that regulate the expression of genes involved in the resistance of the organism to antimicrobial agents can also increase the resistance of the organism. Mutations in *ampR*, the gene that regulates the expression of *AmpC*, results in the overexpression of *AmpC* and thereby increases resistance to beta-lactams (Lister *et al.*, 2009). A number of different resistance genes are encoded on plasmids that have been shown to be acquired by *P. aeruginosa.* These genes either contribute to resistance to the aminoglycosides or to betalactams.

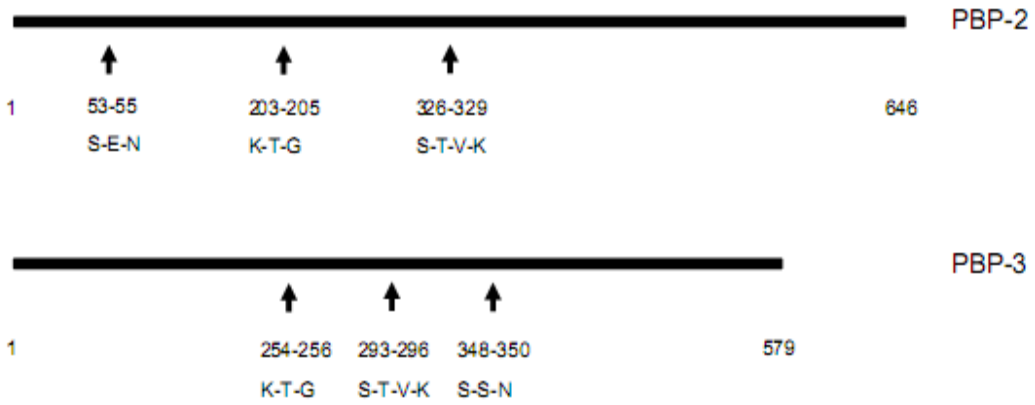


Figure 1.2: The position of the three conserved motifs the active sites of PBP-2 and PBP-3 is demonstrated. Numbers denote amino acid locations in the respective proteins, and letters denote amino acids (S = serine, E = glutamic acid, N = aasparagine, K = lysine, T = threonine, G = glycine, V = valine).

Many beta-lactamases with divergent substrate specificity have been known in several bacterial organisms. *P. aeruginosa* has been shown to produce the following beta-lactamases: Class A extended-spectrum beta-lactamases (ESBL) (*SHV*, *TEM*, *VEB*, *PER* and *CTX-M*), and Class B metallo-beta-lactamases that degrade all beta-lactams including the carbapenems (*IMP*, *VIM*, *SPM*, and *GIM*). *P. aeruginosa* also acquires plasmids encoding aminoglycoside-modifying enzymes (AMEs), as outlined by Llano-Sotelo *et al*. (2002); AMEs can be attached to a phosphate, acetyl or adenyl reactive group to the antibiotic molecule (Llano-Sotelo *et al.*, 2002). Therefore, the binding ability of the modified antibiotics to the target in the microbial cell is reduced. AMEs are plasmid encoded and categorized into three main families; the aminoglycoside N-acetyltransferases (AACs), aminoglycoside O-nucleotidyltransferases (ANTs) and the aminoglycoside O-phosphotransferases (APHs) (Vakulenko & Mobashery, 2003). Chromosomal mutations in the genes encoding the subunits of DNA gyrase and topoisomerase IV are the most significant mechanisms of fluoroquinolone resistance. In accordance to Hooper (2001), there are two common mechanisms, which lead to fluoroquinolone resistance in *P. aeruginosa* (Hooper, 2001). They are structural

modifications in target enzymes and efflux pumps. Alterations in the DNA gyrase, topoisomerase II for fluoroquinolones occur mainly by point mutations in *gyrA*/*gyrB* genes inside the quinolone-resistant-determinative region (QRDR) pattern that is proposed as the enzyme active site. In a bacterial population, these changes exist in low numbers ($1/10^6$ to $1/10^9$). The amino acid changes in GyrA subunit of DNA gyrase regardless of bacterial species are generally localized around the active site where the Tyr122 is covalently linked broken DNA strand during enzyme action (Cabral *et al.*, 1997). Mutations in GyrB and ParE subunit are much less common than those in GyrA and ParC and usually localized to the mid portion of the subunits in a domain involved in interactions with their complementary subunit. The initial step in mutational resistance is achieved by an amino acid change in the most sensitive enzyme generally DNA gyrase in Gram-negative and topoisomerase IV in Gram-positive.

As was pointed out before, four well-known genetically diverse efflux systems have been characterized in *P. aeruginosa:* MexXY–OprM, MexAB–OprM, MexCD–OprJ and MexEF–OprN. Although every pump has preferred antibacterial drug substrates, the fluoroquinolones are common substrates for all of them (Masuda *et al.*, 2000a). Li *et al.* (2003) found a new member of the tripartite multidrug efflux pumps, *MexW* (RND-type membrane protein)-*MexV* (membrane fusion protein) and *OprM* in *P. aeruginosa.* It contributes resistance to chloramphenicol, erythromycin, tetracycline, fluoroquinolones, ethidium bromide and acriflavine (Li *et al.*, 2003b). In *P. aeruginosa* the elevated-level of fluoroquinolone resistance is because of the interaction of mutations in the genes encoding DNA gyrase, topoisomerase IV and the efflux pump systems (Nakajima *et al.*, 2002; Wang *et al.*, 2007). *In vitro*, and *in vivo*, quinolones can select for multidrug-resistant phenotypes (Kohler *et al.*, 1999b). The main and most frequent reasons for their manifestation are the subsequent mutations: *nfxC*, *nfxB* and *nalB* resulting, correspondingly, to the up-regulation of *MexE–MexF–OprN*, *MexC–MexD–OprJ* and

MexA–MexB–OprM (Table 1.2). The newest fluoroquinolones mainly select for *nfxB P. aeruginosa* mutants, whereas older quinolones choose *nfxC* or *nalB* mutants (Kohler *et al.*, 1999b). A phenomenon of antibiotic resistance emerges in *P. aeruginosa* and exhibits all recognized mediated mechanisms of resistance that destroy the activity of antimicrobial agents. Non-enzymatic mediated mechanisms use the efflux pumps, which actively pump the antimicrobial agents out of the cell. In addition, the decreased outer membrane permeability of the cell prevents the antimicrobial agent from getting into the cell (Strateva & Yordanov, 2009; Poole, 2011).

1.7.1 Enzymatic mediated mechanisms of resistance in *P. aeruginosa*

β-Lactamase expression is the most common mechanisms of resistance to β-lactam antibiotics. β-lactamase are able to hydrolyze the four members of the β-lactam antibiotic family including carbapenems, penicillins, cephalosporins and monobactams (Fisher *et al.*, 2005; Henrichfreise *et al.*, 2007). The main cause of acquired resistance to β-lactam antibiotics in *P. aeruginosa* is the β-lactamase expression (Strateva & Yordanov, 2009). In a classification recognized as the Ambler scheme, β-lactamases can be classified depending on the amino acid and nucleotide sequences into 4 major classes: A through D (Ambler, 1980; Paterson & Bonomo, 2005). *P. aeruginosa* clinical isolates express all 4 Ambler classes (Rice, 2006), correlating with the functional classification defined by enzyme substrate and inhibitor profiles (Poole, 2004b). Classes A, C and D act via a serine-based mechanism, while class B or metallo-β-lactamases (MBLs) require zinc for their action. All of these molecular classes of β-lactamases were discovered in *P. aeruginosa*, including ESBLs of classes A, B and C (Beceiro *et al.*, 2004). Classes A, B and C of β-lactamase are the most common and have been discovered worldwide (Beceiro *et al.*, 2004; Yang *et al.*, 1999; Bush & Mobashery, 1998). In the 1990s, class A ESBLs was discovered in the clinical isolates of *P.*

aeruginosa, which involved *TEM*, *SHV, PER*, *VEB*, *GES*/*IBC* and *BEL*. These six types have low identity at the genetic level, and yet they have similar hydrolysis profiles. Class A ESBLs were initially reported in the second half of the 1980s and their dissemination increased since 1995 (Bradford, 2001; Philippon *et al.*, 2002; Ishii *et al.*, 1995). ESBLs are plasmid-encoded enzymes that have mutated from more common β-lactamase enzymes. The presence of ESBL-producing organisms is associated with higher morbidity and mortality than non-ESBL producers (Stein, 2005). Classical ESBLs have developed from the common plasmid-encoded enzyme families cefotaxime (*CTX-M*), *SHV*, *TEM* and oxacillin (*OXA*). The *TEM 1* enzyme was originally found in *E. coli* isolated from a patient named Temoniera and hence was named *TEM* (Medeiros, 1984). TEM 1 is unable to attack the oxyimino cephalosporin but it is able to hydrolyze first generation cephalosporins and penicillins (Sougakoff *et al.*, 1988a; Sirot *et al.*, 1987b). The first *TEM* variant with increased activity against extended spectrum cephalosporins was *TEM 3* which was described in 1983, and now over 100 additional *TEMs* have been characterized (Bradford, 2001; Sougakoff *et al.*, 1988a; Sirot *et al.*, 1987b). Numerous *P. aeruginosa* strains, producing *TEM-24* ESBL were isolated from a long-term-hospitalized woman, including urinary and respiratory isolates (Marchandin *et al.*, 2000). The diversity of *TEM-24*-producing organism recovered from the same patient strongly suggests the *in vivo* horizontal transfer of this plasmid-mediated ESBL from *Enterobacteriaceae* to *P. aeruginosa*.

The *SHV 1* (sulphydryl variable) is the second most common plasmid mediated β-lactamase found in *E. coli* and *K. pneumoniae* (Tzouvelekis & Bonomo, 1999). There are fewer derivatives of *SHV 1*. However, a multiresistant transferable plasmid encoding the *SHV 5* β-lactamase causing unusually high resistance to aztreonam and ceftazidime and a combination of acetylating enzymes producing resistance to all clinically

available aminoglycosides was recognized in *K. pneumoniae* (Galani *et al.*, 2002). In clinical *P. aeruginosa* isolates, *SHV-5* and *SHV-12* genes were identified from Thailand and Greece (Poirel *et al.*, 2004a; Chanawong *et al.*, 2001). *PER-1* is a chromosome encoded gene, identified and completely characterized as ESBL in *P. aeruginosa* and isolated from the urine culture of a Turkish citizen in 1991 in France (Nordmann & Naas, 1994). Currently, among healthcare-associated *P. aeruginosa* isolates the dissemination of bla_{PER-1} is widespread in Poland, Italy, Turkey and Belgium (Kolayli *et al.*, 2005; Empel *et al.*, 2007; Pagani *et al.*, 2004; Claeys *et al.*, 2000). *PER*-1 presents the substrate profile of ESBLs and it is moderately inhibited by β-lactamase inhibitors and imipenem (Weldhagen *et al.*, 2003). The *VEB* enzymes are another type of molecular class A ESBLs. *VEB*-1 β-lactamase was isolated in 1998 in France (Naas *et al.*, 1999). In Thailand, Girlich *et al*. (2002) reported the prevalence of $bla_{VEB-like}$ genes in (93%) ceftazidime-resistant *P. aeruginosa* clinical isolates (Girlich *et al.*, 2002), then identified a new bla_{VEB-2} gene. The only difference between *VEB-1* and *VEB-2* is a single amino acid outside the active site of the enzyme. Recently, the high dissemination (56.8%) of *VEB-1* ESBL was reported from Bulgaria among ceftazidime-resistant healthcare-associated *P. aeruginosa* isolates (Strateva *et al.*, 2007). The VEB enzymes substrate profile was very similar with that of PER-1 (Weldhagen *et al.*, 2003). An ESBL strain is acquired due to contact with a colonized health care worker or contaminated fomites. Hereafter, the ESBL producing strains emerge due to the selective effect of antibiotic use (Lautenbach *et al.*, 2001).

Removal of selective pressure by drug class restriction leads to the disappearance of ESBL producing strains (Urban *et al.*, 2000). The seriously ill patients with prolonged hospital stays, in whom invasive medical devices are present, are considered important factors leading to acquisition and infection with ESBL producing organisms (Paterson & Bonomo, 2005; de champs *et al.*, 1989; Wiener *et al.*, 1999). Heavy antibiotic use is

also a risk factor for acquisition of an ESBL producing organism (Peña *et al.*, 1998). The emergence of ESBLs has been shown to be associated with over use of aminoglycosides, quinolones and third generation cephalosporins ceftazidime (Lee *et al.*, 2003; Rice *et al.*, 1996; De champs *et al.*, 1991). Patient to patient transmission has also been described (Paterson & Bonomo, 2005). However, in a particular unit of a hospital, the same ESBLs may be mediated by different plasmids and many different ESBLs may be found in the same unit at a same time (Bradford *et al.*, 1994). Usually the epicenters of ESBL production are the intensive care units. From hospital to hospital the transfer of genotypically related ESBLs may occur (Monnet *et al.*, 1997), from city to city (Yuan *et al.*, 1998), country to country (Gori *et al.*, 1996) and even intercontinental transfer has been described (Shannon *et al.*, 1990). Community acquired infections with ESBLs have also been found (Rodríguez-Baño *et al.*, 2004).

Genes for class A enzymes *TEM* and *SHV* can be found in the chromosomal DNA of their host bacteria, or on mobile genetic elements called plasmids, which bacteria share with one another. This enhances the spread of resistance among different types of bacteria in both the hospital and general environment. The original or "wild type" *TEM* and *SHV* enzymes were highly suited to hydrolyzing penicillins, with active sites that accommodate the penicillins like a hand in a glove. In contrast to the penicillins, highly active third generation cephalosporins possessed bulky side chains that precluded entry into the *TEM* active site. The widespread use of these "extended-spectrum" cephalosporins in the 1980s was associated with the appearance of the so-called "extended-spectrum beta-lactamases" (ESBLs) (Kliebe *et al.*, 1985; Podbielski *et al.*, 1991; Sirot *et al.*, 1987a; Sougakoff *et al.*, 1988b). Class A ESBLs hydrolyze aztreonam and oxyimino-cephalosporins, but not 7-∝-substituted β-lactams, and are generally susceptible to β-lactamase inhibitors such as sulbactam, clavulanate and tazobactam

(Shahid *et al.*, 2009). Many groups of β-lactamases have hydrolytic profiles similar to those of the *SHV* and *TEM* mutants but have different evolutionary histories. These non-*TEM* and non-*SHV* plasmid mediated class A ESBLs have been sub-divided into ceftazidimases (*PER*, *VEB*, *TLA*-1, and *GES*/*IBC* types) and cefotaximases (*CTX-M*, *SFO*-1, and *BES*-1 type) (Livermore, 2008; Bonnet, 2004). According to recent reports, *CTX-M* β-lactamases are the most widespread ESBL enzymes worldwide (Bradford, 2001; Gniadkowski, 2001). Nearly 113 *CTX-M* variants have been identified and designated from *CTX-M-1* to *CTX-M-113* (http://www.laheyclinic.org/studies/webt.htm). *CTX-M*-producing isolates are now endemic in South America, most European countries and Asia. Recently *CTX-M* enzymes have been detected in a number of focal outbreaks from different parts of the world, e.g., in India (*CTX-M-15*) (Karim *et al.*, 2001), Japan (Toho-2) (Ma *et al.*, 1998) and UK (Naas & Nordmann, 1999) suggesting their wide dispersal.

ESBL-producing isolates are usually selected in hospitals. They may become visible in hospitals due to drug selection, which may lead to a novel type of the enzyme. When selected, the ESBL producers may spread in the hospitals in a number of ways, including clonal dissemination of the producer isolates or horizontal transmission of the ESBL gene-carrying plasmid among non-related isolates (Poirel *et al.*, 2004b; Neonakis *et al.*, 2003). Carbapenem-hydrolysing enzymes are a further group of ESBLs occurring in *P. aeruginosa* and known as MBLs or carbapenemases because of the presence of Zn^{2+} in their active centre (Nordmann & Guibert, 1998).

MBLs or carbapenemases enzymes, mostly classified as Ambler class B metallo-β-lactamases (MBLs), are zinc dependent and have a very wide substrate profile, such as carbapenems and expanded-spectrum cephalosporins. The three subclasses of clinically relevant mobile MBLs that have been identified thus far in *P. aeruginosa* are; *VIM*, *IMP*

and *SPM*-1. A new fourth subclass of Ambler class B enzyme, *GIM*-1, was lately detected in isolates that originated from Germany (Castanheira *et al.*, 2004). Most recent resistances have emerged in Gram-negative bacteria (e.g., *NDM*-1) (Kumarasamy *et al.*, 2010). In *P. aeruginosa,* acquired carbapenemases is an emergent cause of resistance and there has been an increase in the reports on carbapenemases over the last decade (Nordmann & Poirel, 2002).

Carbapenemase production establishes resistance to all β-lactams including the carbapenems (meropenem and imipenem). The hydrolytic features of MBLs do not affect the monobactam aztreonam (Bush & Mobashery, 1998). Metallo-betalactamase (MBLs) production is a significant problem especially in hospital isolates of *P. aeruginosa*. MBLs are spreading throughout various parts of the world. In 1988, it was found that the *IMP* type MBL emerged from a *P. aeruginosa* strain in Japan (Watanabe *et al.*, 1991). However, other genes such as *SPM*, *VIM*, and *GIM* type MBLs were recognized worldwide (Navaneeth *et al.*, 2002; Gupta *et al.*, 2006). Carbapenem non-susceptible, *IMP*-1 producing bacteria were isolated in Europe, Korea and Singapore. In Malaysia, only *IMP-7* gens were found in *P. aeruginosa* (Lagatolla *et al.*, 2004). 11% of the *Pseudomonas spp*. carried bla_{VIM} among imipenem-nonsusceptible isolates reported in Korea (Lee *et al.*, 2004). These bla_{MBL} genes are located on class 1 integrons residing on mobile plasmids. The resistance cassettes carried on these MBL-containing integrons may vary, but this machinery enables resistance to spread horizontally between species in all cases (Rowe-Magnus & Mazel, 2002; Rice, 2002). The genes encoding *IMP*, *VIM* in addition to *GIM* were found as gene cassettes in class 1 integrons, although *IMP* MBL genes have also been found on class 3 integrons (Fluit & Schmitz, 1999). However, not all MBL genes are necessarily associated with integrons or transposons. The genetic context of $bla_{SPM\text{-}1}$ is noticed in mobile genomic pathogenicity islet and is found on a plasmid of 180 kb (Shibata *et al.*, 2003).

Poirel & Nordmann (2002) reported that the class B carbapenem hydrolyzing enzymes activity is not suppressed by tazobactam and clavulanic acid, however it is inhibited by bivalent ionic chelators (Poirel & Nordmann, 2002). Based on Ambler and the first functional group according to Bush, an inducible chromosome-encoded *AmpC* β-lactamase (cephalosporinase) that belongs to molecular class C is produced by *P. aeruginosa* (Bush *et al.*, 1995), *P. aeruginosa* Wild-type strains produce *AmpC* in low quantities (low expression level). *AmpC* is a common chromosomally encoded enzyme found in many Gram-negative bacteria (Campbell *et al.*, 1997; Colom *et al.*, 1995; Hanson & Sanders, 1999). It is inducible by a number of β-lactam antibiotics (e.g., narrow-spectrum cephalosporins and benzyl penicillin) and thus confers intrinsic resistance (Livermore & Brown, 2001), but is not inducible by monobactams (aztreonam), antipseudomonal piperacillin, penicillin (Livermore, 1995) and some of the newer cephalosporins (e.g. ceftazidime, ceftriaxone, cefotaxime) that are, nevertheless, good substrates for the enzyme.

Resistance for these antibiotics is caused by the mutational derepression of *AmpC* (Livermore & Yang, 1987; Poole, 2004b). In fact, mutational derepression of *AmpC* is the most frequent mechanism of resistance to β-lactams in *P. aeruginosa* (Tam *et al.*, 2007; Drissi *et al.*, 2008; Xavier *et al.*, 2010), and this includes expanded-spectrum cephalosporins (e.g., ceftazidime) and penicillins (e.g., ticarcillin) (Dubois *et al.*, 2008; Queenan *et al.*, 2010). While carbapenems (e.g., imipenem) are excellent inducers of *AmpC*, their rapid bactericidal activity and stability to hydrolysis renders them effective against *AmpC* overproduction *P. aeruginosa* (Jones, 1998), while derepressed *AmpC* emerges to confer to carbapenem resistance in association with other mechanisms of resistance (e.g. loss of porin protein D). Lately, the production of *AmpC* variants with improved activity against oxyiminocephalosporins (e.g., ceftazidime), carbapenems

(including imipenem), and cefepime have been reported in clinical isolates of *P. aeruginosa* (Rodríguez-Martínez *et al.*, 2009). These, also, emerge to confer carbapenem resistance in association with loss of *OprD* (Rodriguez-Martinez *et al.*, 2009).

1.7.2 Non-enzymatic mediated mechanisms of resistance in *P. aeruginosa*

There are two types of non-enzymatic mediated mechanisms of resistance in *P. aeruginosa* and they are the efflux pump expression and outer membrane protein. Modification bacterial efflux systems are capable of extruding antibiotics and to date, five families of bacterial drug efflux pumps have been identified. They are the ATP-binding cassette (ABC) family, the major facilitator (MF) family, the small multidrug resistance (SMR) family, the resistance nodulation-division (RND) family, and the multidrug and toxic compound extrusion (MATE) family. The main clinically relevant efflux systems belong to the RND efflux systems because of their abundance and contribution to antibiotic resistance (Poole, 2004a). These efflux pumps usually work as a tripartite system that consist of the cytoplasmic membrane protein that roles to efflux antibiotics through the cytoplasmic membrane, an outer membrane channel protein that supplies the means of access of drugs to the outer part of the cell and a linker protein that joins the two pumps to a membrane fusion protein (MFP) (Livermore, 2002). Together these three proteins are capable of efficiently pumping a range of ions, dyes and antibiotics depending on the type of RND pump combined with the system. There are 12 RND-type efflux systems found in *P. aeruginosa*, however, only four genetically different three-part efflux systems confer resistance to antipseudomonal agents (Livermore, 2001b; Livermore, 2002): MexX-MexY, MexA-MexB-OprM, MexE-MexF-OprN and MexC-MexD-OprJ. The first component is a protein situated in the cytoplasmic membrane (MexY, MexB, MexF and MexD) that functions as a pump and is energy dependent with broad substrate specificity. The second component is a gated

outer membrane protein (OprM, OprM, OprN and OprJ). The third protein (MexX, MexA, MexE and MexC) is positioned in the periplasmic space and links the other two. Efflux pump substrate specificities are summarized in Table 1.2 (Sanchez *et al.*, 1997; Mokhonov *et al.*, 2004; Lister *et al.*, 2009). *MexXY-OprM* and *MexAB-OprM* efflux systems contribute together in acquired and natural antibacterial-resistance mechanisms of *P. aeruginosa*, whereas *MexEF-OprN* and *MexCD-OprJ* only act in acquired resistance (Llanes *et al.*, 2004; Poole *et al.*, 1996a; Kohler *et al.*, 1999a).

In *P. aeruginosa*, the *MexAB-OprM* system was the first pump detected (Li *et al.*, 1995). The MexAB-OprM system has the widest substrate specificity for antimicrobial agents, which include tetracyclines, macrolides, quinolones, chloramphenicol and β-lactams such as, ceftazidime, cefepime, piperacillin, and aztreonam (Li *et al.*,1995; Li *et al.*, 1994; Kohler *et al.*, 1996; Li *et al.*, 1998b). Cao *et al*. (2004) reported that in *P. aeruginosa* the overexpression *MexA–MexB–OprM* might decrease susceptibility to meropenem. There is however no activity on the extra imipenem and carbapenems-panipenem (compared to *P. aeruginosa* wild-strain). This is because the diverse molecular structure of carbapenems-meropenem has a hydrophobic side-chain at the second location, which makes it a substrate for this efflux system. At the same time, panipenem and imipenem are not substrates as their side-chains are hydrophilic and charged (Cao *et al.*, 2004b). Besides this, the substrates include non-antibiotic compounds such as detergents, dyes (ethidium bromide, crystal violet, acriflavine and acridine orange), organic solvents and triclosan (Li *et al.*, 1998a; Li & Poole, 1999). The *MexR* repressor negatively controls the overproduction of the *MexAB-OprM* system. Mutational inactivation of *nalC* regulatory gene increases the expression of the PA3719 protein, which then increases *MexAB-OprM* expression. *nalC* is the second regulatory gene and the mutational inactivation of this regulatory gene leads to increase expression of the PA3719 protein, which then increases the MexAB-OprM

overproduction. *nalD* is the third regulatory gene of the *mexAB-OprM* operon, and mutations in this gene have been noted in clinical isolates that up regulate MexAB-OprM (Hancock & Brinkman, 2002). The MexCD-OprJ system is not expressed constitutively. However, MexCD-OprJ overproduce as have mutations in the *nfxB* gene, which encodes a transcriptional repressor (Poole *et al.*, 1996b). This efflux system mostly exports the antipseudomonal β-lactams (cefepime, piperacillin and meropenem, but not ceftazidime, aztreonam or imipenem), fluoroquinolones, tetracycline, chloramphenicol and macrolides (Li *et al.*, 2000a). MexEF-OprN is the third efflux system, which can confer resistance to quinolones, chloramphenicol and trimethoprim (Kohler *et al.*, 1999c). MexEF-OprN is up regulated by *nfxC P. aeruginosa* mutants. In addition, *nfxC* mutations cause cross-resistance to imipenem due to have down-regulation of OprD outer membrane proteins. Conversely, the MexEF-OprN is subject to regulation by the MexT protein, which belongs to the LysR family of transcriptional activators (Kohler *et al.*,1999c; Li *et al.*, 2000b). Masuda *et al*. (2000) have reported that MexXY-OprM overexpress export fluoroquinolones, aminoglycosides, erythromycin and tetracycline from bacterial cells (Masuda *et al.*,2000a). This is known as 'intrinsic resistance' of *P. aeruginosa* to antipseudomonal agents (Mao *et al.*, 2001). Like MexAB–OprM, MexXY proteins are constitutively overexpressed in stains containing mutations in the *mexZ* repressor gene that is located close and transcribed divergently from the mexXY operon (Hocquet *et al.*, 2007).

The efflux pumps in *P. aeruginosa* are different from each other in various ways, which include the substrate antibiotics they extrude, and regulation of their operons. However, just the mexXY efflux system has been detected to supply both impermeability and adaptive resistance to aminoglycoside antibiotics in these bacteria (Hocquet *et al.*, 2003; Sobel *et al.*, 2003; Vogne *et al.*, 2004). A dominant resistance mechanism in *P. aeruginosa* is the overexpression of efflux systems with wide substrate profiles. The

effect of the overexpression of efflux systems one the resistance to antipseudmonal antibiotics (aminoglycosides, β-lactams, fluoroquinolones and polymyxin B) are summarized in Table 1.3 (Livermore, 2002). In *P. aeruginosa,* the outer membrane works as a protecting permeability barrier, which slows down the diffusion of antibiotics and other toxic compounds into the cell. On the other hand, many substrates essential for the growth of the cell must pass through this permeability barrier and in *P. aeruginosa* this is achieved by the existence of water-filled protein channels identified as porins located in the outer membrane. There are three large families of porins in *P. aeruginosa.* They are: the OprM family of efflux porins, the OprD family of specific porins and the TonB family of gated porins (Hancock & Brinkman, 2002). These porin proteins transport nutrients such as amino acids, sugars and phosphate (Hancock *et al.*, 1990). These porin channels also provide the route for hydrophilic antibiotics such as aminoglycosides, β-lactams, tetracyclines and some fluoroquinolones (Nikaido, 1989; Yoshimura & Nikaido, 1985). The OprD channel's choice for carbapenems is related to the chemical similarity between carbapenems and basic amino acids, permitting binding of the carbapenems to the external loop positions 2 and 3. Therefore, mutations in these positions confer resistance to the carbapenems (Ochs *et al.*, 2000). The external loop positions 5, 7 and 8 are considered to constrict the channel entrance (Huang *et al.*, 1995). In addition, amino acid changes or deletions in these regions have been found to expand the channel, conferring a hyper-susceptible phenotype (Epp *et al.*, 2001).

Even though alterations in the carbapenem-binding regions of OprD will possibly confer a carbapenem resistant phenotype, the main mechanism of carbapenem resistance in *P. aeruginosa* clinical isolates is the down-regulation of OprD (Pai *et al.*, 2001; Livermore, 2001b). Usually, the meropenem MIC is elevated to 2-4 mg/L and imipenem MIC to 8-32 mg/L in isolates characterized by down-regulation of OprD

(Livermore, 2001b). The rate of resistance emerging during imipenem therapy in *P. aeruginosa* was 17% (Calandra *et al.*, 1986). However, another study found a resistance frequency of 44%, which was considered the highest of the antipseudomonal agents (Carmeli *et al.*, 1999a). Some investigators reported that the mechanism by which OprD is decreased is due to the mutation of the structural gene OprD or the putative promoter region, which is responsible for the loss of OprD (Yoneyama & Nakae, 1993; Pirnay *et al.*, 2002; Masuda *et al.*, 1995). The mutations associated with OprD and its promoter, down-regulation of OprD in mutant *P. aeruginosa* is coupled with the up-regulation of MexEF-OprN. These mutants demonstrate up-regulation of the MexEF-oprN during the action of a transcriptional regulator MexT. While a consensus binding region has not been recognized upstream of OprD , expression of cloned MexT from a plasmid has been found to repress the expression of OprD which was adequate to considerably increase MIC of imipenem (Masuda *et al.*, 1995; Ochs *et al.*, 1999).

Table 1.2: Efflux pump substrate specificities of the efflux pump systems in *P. aeruginosa*

Pump Periplasmic linker	Cytoplasmic membrane	Outer membrane channel	Substrates
MexA	MexB	OprM	β-lactams except imipenem, macrolides, tetracycline's, chloramphenicol, novobiocin, Quinolones, lincomycin
MexC	MexD	OprJ	meropenem, macrolides, novobiocin, Quinolones, lincomycin, tetracyclines ,chloramphenicol, penicillins except sulbenicillin and carbenicillin, cefepime, cefpirome
MexE	MexF	OprN	Fluoroquinolones, carbapenems
MexX	MexY	OprM	Quinolones, cefepime, meropenem, cefpirome. aminoglycosides, macrolides, lincomycin, chloramphenicol, tetracycline's and, Penicillins except sulbenicillin and carbenicillin

Table 1.3: Effect of the overexpression of efflux pumps on the resistance to antimicrobial agents

Overexpression of:	Mutation site	Impact on resistance to antipseudomonal with antimicrobial agents activity								
		Fq	CAR TIC	PIP AZL	CAZ ATM	FEP CPO	IMP	MEM	Agl	PB
MexX–MexY–OprM	*mexZ*	r/R	r/R	r/R	r/R	r/R	–	r	r/R	–
MexA–MexB–OprM	*mexR*; PA3721 and PA3719; PA3574	r/R	R	r/R	r/R	r/R	–	r	–	–
MexC–MexD–OprJ	*nfxB*	r/R	r/R	r/R	r/R	R	–	r	–	–
MexE–MexF–OprN	*mexT*	r/R	r/R	r/R	r/R	r/R	r	r	–	–

Aztreonam (ATM,); aminoglycosides(Agl); fluoroquinolones(Fq); azlocillin(AZL); carbenicillin(CAR); ceftazidime(CAZ); cefpirome(CPO); cefepime(FEP); imipenem(IMP); meropenem(MEM); ticarcillin(TIC); piperacillin (PIP); polymyxin B(PB); resistance(R) and reduced susceptibility(r).

1.8 Objectives

This study contributes to the understanding of antibiotic susceptibility and resistance, characterization of the causes of resistance and mechanisms of resistance to assist in the management of infections caused by *P. aeruginosa*.

A number of studies have been performed to determine the effectiveness of several antibiotics to these bacteria (Livermore *et al.*, 2001, Nadeem Sajjad Raja, 2007, Olayinka *et al.*, 2004, Wroblewska *et al.*, 2006).

There are several studies (Islam *et al.*, 2004, Dumas *et al.*, 2006, Strateva *et al.*, 2007, Tam *et al.*, 2009) that evaluate the chromosomally encoded resistance mechanisms among *P. aeruginosa* clinical isolates. However, to date (2009), this is the first study to investigate the contribution of different mechanisms of multidrug resistance among the Malaysian clinical *P. aeruginosa* isolates.

Thus, the purpose of this study was to analyze the expression of multidrug efflux pumps in addition to its relationship with further resistance mechanisms, such as porin down-regulation and β -lactamase production, between *P. aeruginosa* clinical isolates.

Furthermore this study aimed to elucidate whether production of metalo-β-lactamase (MBL) contribute to imipenem resistance in the clinical isolates of *P. aeruginosa*.

The specific objectives of this study are:

1. To identify and characterize *P. aeruginosa* clinical isolates from the University of Malaya Medical Center, Kuala Lumpur, Malaysia.
2. To determine the antibiotic resistance profile for each isolate.
3. To determine the drug resistance genes in these isolates according to the mechanisms of antibiotic resistance among the multidrug resistant isolates.
4. To elucidate whether the production of metallo-β-lactamase (MBL) contributes to imipenem and meropenem resistance in clinical isolates of *P. aeruginosa.*

Chapter 2

Materials and Methods

2.1 Bacterial isolates

Eighty-eight *P. aeruginosa* were collected randomly from April 2009 to March 2010 from the University of Malaya Medical Center, Kuala Lumpur, Malaysia, from various samples. The clinical specimens of *P. aeruginosa* isolates were isolated from urine, wound, sputum, blood and indwelling medical devices. Specimens were collected from different hospital wards such as surgery, general medicine, orthopedics, paediatric, neurosurgery, intensive care units, ENT and gynaecology wards. The control strain used in this study was *P. aeruginosa* ATCC 27853.

2.1.1 Cultivation of clinical specimens

10 mL of blood from a venopuncture was collected into a Bactec Plus Aerobic/F (gray cap) bottle and incubated in a BACTEC 9000 Series blood culture instrument (Becton Dickinson, USA). The positive sample was then subcultured onto blood agar. Sputum was considered acceptable for the culture if it contained <25 epithelial cells per low-power field and >25 polymorphonuclear leukocytes. Sputum was then cultured on chocolate agar, blood agar and macconkey agar. In addition, body fluids and wound samples were cultured on chocolate agar, blood agar and MacConkey agar and then incubated in a 5% CO_2 incubator at 37°C for 24 hours. In dwelling medical devices and urine samples were cultured on MacConkey agar and blood agar then incubated at 37°C in an incubator for 24 hours.

2.1.2 Preservation of isolates

To store the isolates for the long-term, isolates were subcultured on Nutrient agar and incubated at 37°C for 24 hours. A single colony of pure culture was then cultured on LB broth (Luria Bertani) and incubated overnight. The bacterial cell pellet was transferred to the 1.5 mL plastic tubes and LB broth (Luria Bertani) in addition to 30% V/V glycerol were added. They were then kept frozen at -80°C until used. *P. aeruginosa*

isolates were then recovered from frozen stocks when needed. This was done by subculturing on Nutrient agar and incubation for 24 hours in a 37°C incubator.

2.1.3 Ethical consideration

The protocol for this study (Reference Number: 902.5) and use of isolates has been approved by the Medical Ethics Committee of the University of Malaya Medical Centre, Kuala Lumpur, Malaysia (Appendix I).

2.2 Conventional methods of identification and characterization of *P. aeruginosa*

P. aeruginosa colonies were identified based on morphology, Gram staining, pyocyanin production and a biochemical test by API20NE (bioMérieux, Marcy-l'Etoile, France). using standard laboratory procedures (Binnerup *et al.*, 1998).

2.2.1 Gram staining

The Gram stain was carried out on 24 hours cultures according to the Hucker method (Binnerup *et al.*, 1998). Three to four colonies of pure *P. aeruginosa* culture were smeared thinly on to a glass slide and air-dried, then it was fixed with a gentle flame heat or 95% methanol was added for 2 minutes. The primary stain (Crystal violet) was added and allowed to stand for 1 minute. Excess stain was washed off with a gentle stream of tap water. The Gram's iodine was added and allowed to stand for 30 seconds before being rinsed off. This was decolorized by acetone for 5 to 15 seconds, then washed with tap water, then stained with the counter stain Safranin for 1 minute, and washed with tap water. Finally, the slide was air dried prior to being examined under a microscope. If the bacteria were Gram-negative, they appeared pink under the microscope. If the cell was Gram-positive, it appeared purple under the microscope.

2.2.2 Motility test

Motility is demonstrated macroscopically by making a straight stab of pure *P. aeruginosa* colonies grow in nutrient broth. This was incubated for 24 hours at 37°C, and then the *P. aeruginosa* grown in nutrient broth was placed on a slide and covered with a clean cover slip. The motility examination was then done by a microscope (Barrow & Feltham, 2004).

2.2.3 Oxidase test

The filter paper was saturated with oxidase reagent (bioMérieux, Marcy-l'Etoile, France) that was placed in a petridish. A loopful of *P. aeruginosa* from a 24-hours-old culture was inoculated onto filter paper. Development of a blue color at the site of inoculation within 10 to 30 seconds indicated a positive reaction (Barrow & Feltham, 2004).

2.2.4 Biochemical tests

P. aeruginosa identification was performed using the commercially available kit, API 20NE (bioMérieux, Marcy-l'Etoile, France) according to the manufacturer's instructions. It consisted of 20 microtubes containing dehydrated substrates as is illustrated in Table 2.1. The control strain used in this study was a *P. aeruginosa* ATCC 27853 (Figure 2.1).

The analysis of data was done by using the API20NE identification software that was provided by the manufacturing company.

Table 2.1: Biochemical test demonstrating the API20NE parameters and the Characteristics of *P. aeruginosa*

TESTS	ACTIVE INGREDIENTS	Biochemical tests	Characteristics of *P. aeruginosa*
NO3	potassium nitrate	reduction of nitrates to nitrites reduction of nitrates to nitrogen	Positive
TRP	L-tryptophane	indole production (TRyptoPhane)	Negative
GLU	D-glucose	fermentation (GLUcose)	Negative
ADH	L-arginine	Arginine DiHydrolase	Variable
URE	urea	UREase	Variable
ESC	Esculin ferric citrate	hydrolysis (β-glucosidase) (ESCulin)	Negative
GEL	Gelatin (bovine origin)	hydrolysis (protease) (GELatin)	Positive
PNPG	4-nitrophenyl-β-Dgalactopyranoside	β-galactosidase (Para-Nitro Phenyl-ßDGalactopyranosidase)	Negative
GLU	D-glucose	assimilation (GLUcose)	Positive
ARA	L-arabinose	assimilation (ARAbinose)	Negative
MNE	D-mannose	assimilation (ManNosE)	Negative
MAN	D-mannitol	assimilation (MANnitol)	Positive
NAG	N-acetyl-glucosamine	Assimilation (N-Acetyl-Glucosamine)	Positive
MAL	D-maltose	assimilation (MALtose)	Negative
GNT	potassium gluconate	assimilation (potassium GlucoNate)	Positive
CAP	capric acid	assimilation (CAPric acid)	Positive
ADI	adipic acid	assimilation (ADIpic acid)	Positive
MLT	malic acid	assimilation (MaLaTe)	Positive
CIT	trisodium citrate	assimilation (trisodium CITrate)	Positive
PAC	phenylacetic acid	assimilation (PhenylACetic acid)	Negative
OX	oxidase test	cytochrome oxidase	Positive

Figure 2.1: API 20NE tests that consisted of 20 microtubes containing dehydrated substrates.

2.3 Antibiotic susceptibility testing

The susceptibility of various antibiotics against *P. aeruginosa* clinical isolates was determined using an E-test® (bioMérieux) according to the guidelines of the Clinical and Laboratory Standards Institute (Tholen, 2006). Mueller-Hinton agar was prepared as follows. The agar was prepared according to the manufacturer's instructions to a pH of 7.2-7.4. Immediately after autoclaving, the agar was allowed to cool to 45-50°C. The freshly prepared agar was poured into plastic, flat-bottomed petri dishes on a level surface to give a uniform depth of approximately 4 mm (this corresponded to 25-30 mL of agar in a plate with a diameter of 100 mm). The plates were allowed to cool to room temperature and, unless the plates were used on the same day, they were stored at 2-8°C in a refrigerator. All plates were used within seven days of preparation. A representative

sample of each batch of plates was examined for sterility by incubating at 37°C for 24 hours.

The E-test® was used as a quantitative antimicrobial susceptibility testing method. This included the equal diffusion and dilution of antibiotics into the medium. It is recognized as the epsilometer test as 'E' refers to the Greek sign epsilon (ε). A thin inert strip is a carrier that predefines the antimicrobial gradient. There is an immediate release of the drug when the E-test® strip is applied onto an inoculated agar plate following incubation, as a regular inhibition ellipse is produced. The calibrated carrier strip and the intersection of the inhibitory zone edge indicates the MIC value (Lalitha *et al.*,1997). The antimicrobial agents tested in this study were piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem, meropenem and colistin.

The MIC for each antibiotic was determined on Mueller-Hinton agar. Overnight cultures of *P. aeruginosa* on Mueller-Hinton broth were diluted to an initial cell density of 10^7 cfu/mL with fresh Mueller-Hinton broth. Inoculums were 10^5 cfu to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard. The 0.5 McFarland standard was prepared as follows; a 0.5 mL aliquot of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2$. 2 H_2O) was added to 99.5 mL of 0.18 mol/L H_2SO_4 (1% V/V) with constant stirring to maintain a suspension. The correct density of the turbidity standard was verified by using a spectrophotometer with a 1 cm light path and matched cuvette to determine the absorbance. The absorbance at 600 nm was between 0.08 and 0.1 for the 0.5 McFarland standards.

A sterile cotton swab was dipped into the adjusted suspension, optimally within 15 minutes after adjusting the turbidity of the inoculum suspension. The swab was rotated several times and pressed firmly on the inside wall of the tube above the fluid level to remove the excess inoculum from the swab. The dried surface of the Mueller Hinton

agar plate was inoculated by streaking the swab over the entire sterile agar surface. This procedure was repeated two more times, rotating the plate approximately 60° each time to ensure an even distribution of inoculum. The rim of the agar was then swabbed as a final step. The cover was left partly open for three to five minutes to allow any excess surface moisture to be absorbed before applying E-test® antimicrobial strips. Two different E-test® antimicrobial strips were placed in opposite gradient directions on a Mueller-Hinton agar plate with sterile forceps. Printed MIC values faced upward (i.e., the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar). The plates were incubated in an inverted position at 37°C for 24 hours. After incubation, the MICs were read in the intersection point (break point) of inhibitory eclipse and interpreted according to CLSI standards (Tholen, 2006) and were reported as being susceptible, intermediate or resistant to the agents that were tested. *P. aeruginosa* ATCC 27853 were utilized as quality control strains. The break point of inhibition of each antibiotic were also obtained for the control strains *P. aeruginosa* ATCC 27853 to ensure the method was being performed correctly and compared to the expected point of inhibition according to CLSI (Table 2.2).

Table 2.2: Summary of E-test® interpretative criteria of minimum inhibition concentration (MIC) break point for *P. aeruginosa* according to CLSI (Tholen, 2006)

Antibiotic	Code	MIC range µg/mL	S ≤	I	R ≥	*P. aeruginosa* ATCC 27853 MIC µg/mL
Gentamicin	GM	0.064-1024	4	5	8	1 - 4
Amikacin	AK	0.016- 256	16	17	32	1 - 4
Ciprofloxacin	CI	0.002- 32	1	2	4	0.25-1
Imipenem	IP	0.002- 32	4	8	16	1 - 4
Meropenem	MP	0.002- 32	4	8	16	0.25-1
Ceftazidime	TZ	0.016- 256	8	16	32	1 - 4
Aztreonam	AT	0.016- 256	8	16	32	2-8
Piperacillin*/ Tazobactam**	PTC	0.016- 256* (4 µg/mL)**	16	32-64	128	1-8
Colistin	CO	0.016- 256	2	4	8	0.5 - 4

S: susceptible, I: intermediate or R: resistant.

2.4 Gene expression of *P. aeruginosa* using real time PCR

2.4.1 Extraction and quantitation of RNA

TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) was prepared containing 15 mg/mL lysozyme which was used in the lysis of the bacterial cell wall. The RNAprotect Bacteria Reagent (Qiagen, Hilden, Germany) is a kit used to stabilize RNA before the bacterial cells were lysed. This allows sufficient time for efficient disruption of cells without the risk of distorting the gene expression profile. After cell lysis, the RNeasy Mini Kit was used (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Aliquot (0.25 mL) of the suspended culture (cells pellet suspension), equivalent to 5×10^{8}cfu/mL, was added to 0.5 mL of the RNAeasy bacteria protect solution (Qiagen, Hilden, Germany) then mixed immediately by vortexing for 5 seconds and incubated for 5 min at room temperature (15–25°C), then centrifuged for 10 minutes at 8000 rpm after that supernatant was decanted by gently dabbing the inverted tube once onto a paper towel until the residual supernatant was removed. Then 100 µL of TE buffer containing lysozyme 15 mg/mL (Sigma Aldrich, Germany) was added and vortexed for 10 seconds then incubated at room temperature for 5 minutes. This was also vortexed for 10 seconds every 2 minutes during incubation. In addition, 350 µl of RLT buffer was then added and mixed vigorously by vortex, 96% ethanol 250 µL was added and homogenized well by pipetting. Then 700 µL lysate was transferred to the RNeasy spin column and centrifuged for 15 seconds at 10,000 rpm. The flow through was discarded of and the RNeasy collection tube reused and 350 µL of RW1 buffer was added into a spin column and centrifuged for 15 seconds at 10,000 rpm. Residual DNA was treated with DNase 1U/µL (Qiagen, Hilden, Germany). DNase I was prepared as a stock solution by adding 10 µL of DNase I plus 70 µL of RDD buffer (Qiagen, Hilden, Germany) then mixed gently and centrifuged. The mixture was called DNase I mix, 80

µL of DNase I mix was added to the RNeasy spin column membrane, and incubated at room temperature for 15 minutes, 350 µL of RW1 buffer was added to the spin column, incubated for 5 minutes at room temperature then centrifuged for 15 seconds at 10,000 rpm. The flow-through and collection tube were discarded and the mini spin column was transferred to another 2 mL collection tube, 500 µL of RPE buffer was then added to the mini spin column and centrifuged for 15 seconds at 10,000 rpm. The flow-through was discarded and the collection tube reused. Then500 µL of RPE buffer was added to the mini spin column and centrifuged for 2 minutes at 10,000 rpm. The spin column was placed into another collection tube and centrifuged for a minute at full speed and the mini spin column was placed in a 1.5 mL collection tube. The RNA was then eluted by adding 50 µL of RNase-free water directly and centrifuged for 1 minute at 10,000 rpm. The RNA concentration was measured using NanoDrop-2000. To exclude the possibility of any contamination of the RNA, samples with genomic DNA real time PCR was performed. The extracted RNA was kept at −80ºC until used. The concentration and purity of RNA was determined by measuring the absorbance at (A260) nm and (A280) nm using Nano Drop spectrophotometer (Nano-Drop 2000 Thermo Fisher Scientific, USA) to estimate the ratio between the absorbance values at 260 and 280 nm to ensure the RNA purity. In addition, the ratio of absorbance values at 260 and 230 nm was estimated. For all samples, the ratios of A 260/280 and A 260/230 were above 2.0 in nuclease free water (Figure 2.2).

The integrity and size distribution of the total RNA was checked by 1.2% (w/v) formaldehyde-denaturing agarose gel electrophoresis at 100 V for 50 min (Figure 2.3).

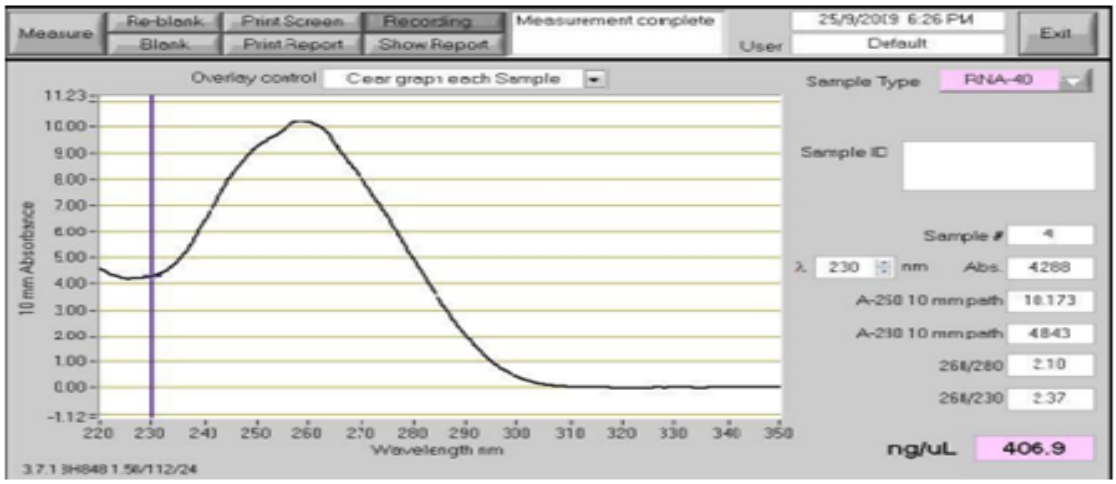
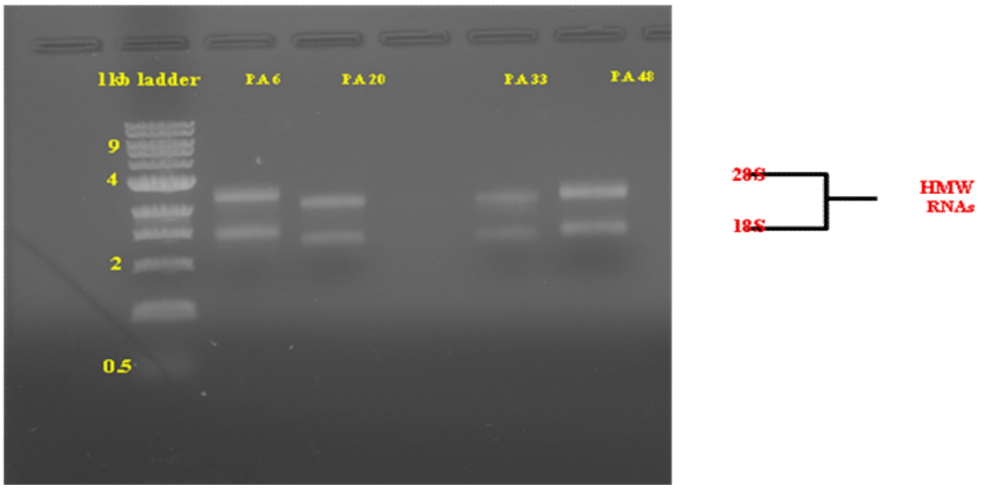


Figure 2.2: Representative diagram of extracted RNA measured by NanoDrop ND-2000. Note the A260/280 is 2.10.

Figure 2.3 Representative picture of 1.2% (w/v) formaldehyde-denaturing agarose Gel electrophoresis to verify the quality of extracted RNA.



2.4.2 cDNA synthesis

500 ng of RNA from all strains was converted to cDNA using a QuantiTect Rev. Transcriptase Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The following steps were taken: The reagents of reverse transcription were thawed at room temperature, then mixed by flicking the tubes, centrifuged briefly, and stored on ice. The RNA was thawed on ice.

The genomic DNA elimination kit was thawing on ice, then the component added as in Table 2.3. This was mixed well and incubated for 10 minutes at 40°C, then placed immediately on ice. The reverse-transcription master mix was prepared on ice according to Table 2.4.

The RNA from step 3 (14 µL) was templated and added to each tube containing the reverse-transcription master mix, and then mixed well. This was incubated in a thermal cycler (Master cycler Gradient PCR, Eppendroff) under the following conditions: 15 minutes at 42°C, then 3 minutes at 95°C, ending at 4°C. The cDNA was kept at –20°C until used.

Table 2.3: Concentration of each reagent that was used to eliminate genomic DNA

Component	Volume/reaction	Final concentration
gDNA Wipeout Buffer, 7x	2 µL	
RNase-free water	Variable	1x
Template RNA	Variable (500 ng)	
Total volume	14 µL	-

Table 2.4: Reverse-transcription reaction components in final volume (20 µL)

Constituent	Volume/reaction	Final concentration
Master mix of reverse-transcription		
Quantiscript RT buffer	4 µL	
Quantiscript reverse transcriptase	1 µL	1x
RT primer mix	1 µL	
Entire genomic DNA wipe out reaction (step 3)	14 µL (added at step 6)	
Total volume	20 µL	-

2.5 Real time PCR to determine expression levels of antibiotic resistant genes

Transcriptional levels of *mexB*, *mexY, mexCD, mexF*, *mexZ, oprM, ampC*, *PBP2*, *PBP3* and *oprD* were determined using gene-specific primers with real-time PCR (Table 2.5) in a CFX96™ PCR detection system (BioRad, Hercules, CA). Amplifications were carried out in triplicate PCR reactions using the iQ™ SYBR® Green supermix (BioRad). The reagent composition of PCR mixtures is presented in Table 2.6. The amplification conditions for real-time PCR of each gene were as shown in Table 2.7. The amplicon for each of the melting curves was evaluated and contrasted with the melting temperature (Tm) obtained when using the cDNA template to ensure that specific amplification had occurred.

The transcriptional levels of the target genes were normalized using a reference gene, *rpoD*. This gene is constitutively expressed in *P. aeruginosa* (Savli*, et al.*, 2003) and experiments showed a stable expression of *rpoD* in all isolates. Normalized expression of each target gene was standardized against corresponding mRNA expression in the *P. aeruginosa* wild strain (ATCC 27853). All published equations and available models for all calculations of the relative expression ratio allow only for the determination of a single transcription difference between one control and one sample. Therefore, the relative gene expression results are presented as ratios between the target gene (target) and the reference gene (*rpoD*), which were obtained based on the following equation:

$$\text{Ratio} = (E_{\text{target gene}})^{\triangle ct}{}_{\text{target (ATCC 27853-sample)}} / (E_{\text{rpoD}})^{\triangle ct}{}_{\text{rpoD (ATCC 27853-sample)}}$$

(Pfaffl *et al.*, 2002), where E is the real-time PCR efficiency for a given gene, △ unknown sample versus a control ($\triangle ct_{\text{ATCC 27853-sample}}$) and C_t the crossing point of the amplification curve with the threshold. Transcription data was analyzed using Bio-Rad CFX manager software.

According to previous studies (Dumas *et al.*, 2006; Savli *et al.*, 2003; Li & Nikaido, 2009; Tomas *et al.*, 2010; Yoneda *et al.*, 2005), the efflux system genes *mexY* and *mexB*

were considered up regulated when the transcription levels of *MexY* and *MexB* were, respectively, at least 4 and 2 fold higher than the reference strain *P. aeruginosa* wild-type (ATCC 27853). Overexpression of *ampC* and down-regulation of *oprD* was considered significant when their transcriptional levels were respectively, ≥ 10 and ≤ 70% -fold, compared to the reference strain wild type. The data were subjected to statistical analysis using SPSS version 11.5 (SPSS Inc., Chicago, US). A chi-square was done to show the mRNA expression in comparison to antibiotics resistance. A *p*-value of ≤ 0.05 was considered indicative of a statistically significant difference. Fisher's exact test is to be used if less than 50% of the cells are less than 5.

Table 2.5: Primers used in real-time PCR

Genes		5′- sequence- 3′	Reference
mexB	F	5′- GTGTTCGGCTCGCAGTACTC - 3′	(Dumas, *et al.*,2006)
	R	5′- AACCGTCGGGATTGACCTTG - 3′	
mexY	F	5′- CCGCTACAACGGCTATCCCT - 3′	(Dumas, *et al.*,2006)
	R	5′- AGCGGGATCGACCAGCTTTC - 3′	
mexZ	F	5′- AGGTCTGCCTGGCGATGTGC- 3′	(Yoneda *et al.*, 2005)
	R	5′- AGCGTTGCCCCTGCTTCTCG – 3′	
oprD	F	5′-CGACCTGCTGCTCCGCAACTA - 3′	(Yoneda, *et al.*, 2005)
	R	5′- TTGCATCTCGCCCCACTTCAG- 3′	
ampC	F	5′- AGATTCCCCTGCCTGTGC- 3′	(Dumas, *et al.*, 2006)
	R	5′-GGCGGTGAAGGTCTTGCT- 3′	
PBP2	F	5′ -GCCCAACTACGACCACAAG - 3′	(Yoneda, *et al.*, 2005)
	R	5′ -CGCGAGGTCGTAGAA ATA G - 3′	
PBP3	F	5′ -TGATCAAGTCGAGCAACGTC - 3′	(Yoneda, *et al.*, 2005)
	R	5′ -TGCATGACCGAGTAGATGGA - 3′	
rpoD	F	5′-GGGCGAAGAAGGAAATGGTC- 3′	(Savli, *et al.*, 2003)
	R	5′ -CAGGTGGCGTAGGTGGAGAA- 3′	
mexCD	F	5′ -GGAGTTCGGCCAGGTAGTGCTG- 3′	(El Amin *et al.*, 2005)
	R	5′ -ACTGCATGTCCTCGGGGAAGAA- 3′	
mexEF	F	5′ -CGCCTGGTCACCGAGGAAGAGT- 3′	(Giske *et al.*, 2005)
	R	5′ -TAGTCCATGGCTTGCGGGAAGC- 3′	
oprM	F	5′ -GATCCCCGACTACCAGCGCCCCG- 3′	(Dumas, *et al.*, 2006)
	R	5′ -ATGCGGTACTGCGCCCGGAAGGC- 3′	

Table 2.6: Reagents used in a real-time PCR master mix for the *MexB*, *MexY, mexCD, mexEF*, *mexZ, oprM, ampC*, *PBP2*, *PBP3* and *oprD* genes (final volume 25 µL)

Reagent	Volume(µl):1sample	Final concentration
iQ™ SYBR® Green supermix	12.5 µL	1X
Forward primer (10 pM)	1.0 µL	1 pM
Reverse primer (10 pM)	1.0 µL	1 pM
Total cDNA (1:4)	2.0 µL	125 ng
RNase-free water	8.5 µL	-
Total volume	25 µL	

Table 2.7: qRT-PCR conditions individual resistance genes

Each step of qRT-PCR	qRT-PCR condition (°C, minutes, seconds)										
	mexB	*mexY*	*mexCD*	*mexEF*	*mexZ*	*oprM*	*ampC*	*PBP2*	*PBP3*	*oprD*	*rpoD*
Predenaturing	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min	95°C 10min
	35 cycles of										
Denaturing	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec	95°C 20 sec
Annealing	58°C 30 sec	59°C 30 sec	63.5°C 30 sec	60°C 30 sec	57 °C 30 sec	63°C 30 sec	56°C 30 sec	64°C 30 sec	62°C 30 sec	61°C 30 sec	55°C 30 sec
Extension	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec	72°C 30 sec
	Plate read										
Final Extension	72ºC 5 min	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5 min	72°C 5min	72°C 5 min
Melting curve 65°C to 95°C, increment 5°C for 0:05 + Plate read											

2.6 Gene identification of metallo-β-lactamase production in *P. aeruginosa* using polymerase chain reaction (PCR)

2.6.1 DNA extraction and quantitation

P. aeruginosa clinical isolates were subcultured on nutrient agar for 24 hours at 37°C. A single colony of pure culture was cultured on LB (Luria Bertani) broth at 37°C for 18 hours and the cells were diluted in fresh LB broth (1:100). They were then grown to logarithmic phase (0.5 at OD_{600}) and the cells pellet was collected by centrifugation at 4000 rpm for 10 minutes at 4°C. The supernatant was removed and the cells pellet was resuspended. The DNA was then extracted using a DNeasy blood and tissue mini kit (Qiagen) according to the manufacturer's instructions using the following steps: The supernatant was removed and the cell pellet resuspended. ATL buffer (180 µL) was added to the cell pellet. Then, 20 µL proteinase K was added and mixed well, then incubated at 56°C for 1 hour. RNase A 4 µL (100 mg/mL) was added and mixed by vortex then incubated for 2 minutes at room temperature to digest genomic DNA. AL buffer (200 µL) was added and mixed thoroughly by vortex. Ethanol (200 µL /96%) was then added and mixed again thoroughly by vortex. The mixture from step 5 was transferred into the mini spin column tube then centrifuged at 8000 rpm for 1 minute. The flow-through and collection tube were discarded. The mini spin column was placed in another collection tube. Then 500 µL AW1 buffer was added then centrifuged for 1 minute at 8000 rpm. The flow-through and collection tube were discarded. The mini spin column was placed in another collection tube. Then 500 µL AW2 buffer was added then centrifuged for 3 minutes at 14,000 rpm to dry the membrane of the mini spin column. The flow-through and collection tube were discarded**.** The mini spin column was placed in a clean 1.8 mL tube and centrifuged at 14,000 rpm for 1 minute to eliminate residual ethanol. The mini spin column was placed in a clean 1.8 mL tube.

The 100 µL AE buffer was added directly and incubated at room temperature for 5 minutes and centrifugation at 8000 rpm was done after that for 1 minutes to elute for DNA. The DNA was stocked up at -20°C until used.

The concentration and purity of DNA was determined by measuring the absorbance at (A260) nm and (A280) nm using a Nano drop-2000 spectrophotometer to estimate the ratio between the absorbance values at 260 and 280 nm to ensure DNA purity. In addition, the ratios between the absorbance values were estimated at 260 nm and 230 nm. The ratios for all samples of A 260/280 and A 260/230 were above 2.0 in nuclease free water.

2.6.2 PCR amplification of the (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$ and *bla*$_{NDM}$) genes

Sixty-five *P. aeruginosa* isolates that were resistant to imipenem and meropenem were tested for MBL production according to the Pitout *et al*. method (Pitout *et al.*, 2005). The genes encoded for the following metallo-β-Lactamase class B (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$ and *bla*$_{NDM}$) were determined from genomic DNA using gene-specific primers for the metallo β-Lactamase genes by PCR amplification assay (Table 2.8).

The amplification was carried out using a Master cycler Gradient PCR (Eppendroff) and top taq™ master mix 250 unit kit (Qiagen). This was used for genes identification (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$ and *bla*$_{NDM}$) and the concentration of each reagent used (Table 2.9) in a final volume of 25 µL. The PCR conditions included the primary incubation at 37°C for 10 minutes and the first step denaturation for 5 minutes at 94°C. This was followed by 30 cycles of DNA denaturation for 1 minute at 94°C, annealing temperature for 1 minute at 54°C, and an extension for 1.5 minutes at 72°C. Finally, the PCR products were analyzed (1/10 volume) by electrophoresis with 2.0% (w/v) agarose gels in a TAE buffer using sybr green dye for 50 minutes at 100 V. The PCR product was visualized with UV light in a gel doc XR system (BioRad).

Table 2.8: The oligonucleotide PCR primers used for identification of Metallo-β-Lactamase Genes

Genes		5´- sequence- 3´	Reference	bp*
bla_{IMP}	F	5-GAA GGY GTT TAT GTT CAT AC-3	(Pitout, *et al.*,2005)	587
	R	5-GTA MGT TTC AAG AGT GAT GC-3		
bla_{VIM}	F	5-GTT TGG TCG CAT ATC GCA AC-3	(Pitout, *et al.*,2005)	382
	R	5-AAT GCG CAG CAC CAG GAT AG-3		
bla_{SIM}	F	5-TACAAGGGATTCGGCATCG-3	(Poirel *et al.*,2011)	570
	R	5-TAATGGCCTGTTCCCATGTG-3		
bla_{SPM}	F	5-AAAATCTGGGTACGCAAACG-3	(Poirel, *et al.*,2011)	271
	R	5-ACATTATCCGCTGGAACAGG-3		
bla_{GIM}	F	5-TCGACACACCTTGGTCTGAA-3	(Poirel, *et al.*,2011)	477
	R	5-AACTTCCAACTTTGCCATGC-3		
bla_{NDM}	F	5-GGTTTGGCGATCTGGTTTTC	(Poirel, *et al.*,2011)	621
	R	CGGAATGGCTCATCACGATC		

**Amplicon size in base pair*

Table 2.9: PCR mixture and concentration of each reagent used for gene identification of (bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{SIM}, bla_{SPM} and bla_{NDM}) in a final volume 25 µL

Reagent	Volume(µL):1sample	Final concentration
top taq™ master mix	12.5 µL	1X
Total DNA	2.0 µL	100-500 ng
Forward primer (10 pM)	1.0 µL	1 pM
Reverse primer (10 pM)	1.0 µL	1 pM
RNase-free water	8.5 µL	-
Total volume	25 µL	

2.6.3 PCR amplification of the beta-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{VEB}$ and *bla*$_{PER}$)

Sixty-five isolates of *P. aeruginosa*, which showed resistance to meropenem and imipenem, were evaluated for the ESBL production genes. The genes encoding for the beta-lactamases (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{VEB}$ and *bla*$_{PER}$) were identified using the conventional PCR amplification assay from genomic DNA using gene-specific primers (Table 2.10). A top taq™ master mix 250 unit kit (Qiagen) was used in a final volume reaction of 50 µL as is illustrated in Table 2.11.

PCR amplification conditions included the first step of denaturation for 15 minutes at 95°C. This was followed by 30 cycles of denaturation for 30 seconds at 94°C, annealing temperature for 30 seconds at 50°C for *bla*$_{TEM}$, at 52°C for *bla*$_{VEB}$, at 54°C for *bla*$_{PER}$, 56°C for *bla*$_{SHV}$ and 58°C for *bla*$_{CTX-M}$ primers. This was followed by extension temperature for 2 minutes at 72°C and a final step extension for 10 minutes at 72°C. Finally, the cycles was hold at 4°C. Subsequently the PCR products (1/10 volume) were analyzed by electrophoresis with 2.0% (w/v) agarose gels in TAE buffer using sybr green dye for 50 minutes at 100 V and visualized with UV light using a gel doc XR system (BioRad).

The whole genome amplified DNA was obtained from the *Klebsiella pneumoniae* strains. Both strains possessing the (*bla*$_{SHV}$, *bla*$_{TEM}$ and *bla*$_{CTX-M}$) gene were kindly provided by Dr. Hans-Jürg Monstein (Monstein *et al.*, 2007), (Clinical Microbiology, Molecular Biology Laboratory, University Hospital, Linköping, Sweden) and used as positive control strains.

Table 2.10: Primers used in PCR amplification for identification of the (*bla*$_{SHV}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{VEB}$ and *bla*$_{PER}$) genes respectively

Genes		5′- sequence- 3′	bp*	reference
bla$_{SHV}$	F	5′- ATGCGTTATATTCGCCTGTG - 3′		(Paterson *et*
	R	5′- TGCTTTGTTATTCGGGCCAA - 3′	747	*al.*,2003)
bla$_{TEM}$	F	5′- TCGCCGCATACACTATTCTCAGAATGA - 3′		(Monstein, *et*
	R	5′- ACGCTCACCGGCTCCAGATTTAT - 3′	445	*al.*,2007)
bla$_{CTX-M}$	F	5′- ATGTGCAGYACCAGTAARGTKATGGC - 3′		(Boyd *et al.*,2004)
	R	5′TGGGTRAARTARGTSACCAGAAYCAGCGG-3′	593	
bla$_{VEB}$	F	5′- CGACTTCCATTTCCCGATGC- 3′		(Naas *et al.*,2000)
	R	5′- GGACTCTGCAACAAATACGC- 3′	643	
bla$_{PER}$	F	5′- AATTTGGGCTTAGGGCAGAA- 3′		(Claeys *et*
	R	5′- ATGAATGTCATTATAAAAGC- 3′	925	*al.*,2000)

**Amplicon size in base pair*

Table 2.11: PCR mixture and concentration of each reagent used for gene identification of (*bla*$_{IMP}$, *bla*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$ and *bla*$_{NDM}$) in a final volume 25 µL

Component	Volume(µL):1sample	Final concentration
top taq™ master mix	20 µL	1X
Forward primer (10 pM)	1.0 µL every gene	1 pM
Reverse primer (10 pM)	1.0 µL every gene	1 pM
Total DNA	4.0 µL	100-500 ng
RNase-free water	20 µL	-
Total volume	50 µL	

Chapter 3

Results

3.1 Characterization of clinical bacterial isolates

Eighty-eight *P. aeruginosa* isolates were collected from various clinical samples from different wards at the University of Malaya Medical Center, Kuala Lumpur, Malaysia. These samples were sourced from April 2009 to March 2010. Preliminary identification tests were performed for all isolates using a variety of techniques, which included morphological characteristics, Gram staining, pyocyanin production and a biochemical test, by API20NE. The clinical specimens of *P. aeruginosa* isolates were isolated from urine (53.4%), wound (21.6 %), sputum (5.7%), blood (5.7%) and indwelling medical devices (13.6 %) as shown in Table 3.1a. Samples were collected from patients hospitalized in the surgical (31), medical (20), orthopedic (13), paediatric (7), neurosurgery (7), intensive care unit (ICU) (5), otorhinolaryngology (ENT) (3) and gynaecology wards (2). Of these isolates, 47 were from urine, 19 from wounds, 12 from in dwelling medical devices, 5 from blood, and 5 from sputum (Table 3.1b).

The morphology of the identified colonies was smooth and large with an elevated center giving it the appearance of a fried egg. Colonies were pigmented blue green due to pyocyanin pigment on the Nutrient agar culture at 37°C as illustrated in Figure 3.1a. When the colonies were cultured on MacConkey agar, they appeared as non-lactose fermenters, with smooth, large, elevated and occasionally mucoid colonies as shown in Figure 3.1b**.** All strains were identified as being Gram negative, rod or coccobacilli shape, and non-spore forming (Figure 3.1c). Further identification was performed using the commercially available identification kit, API 20NE (bioMérieux) according to the manufacturer's instructions. It consisted of 21 microtubes containing enzymatic and carbon compound assimilation tests and all of the isolates were identified as *P. aeruginosa* as shown in Appendix III.

Table 3.1a: the distribution of *P. aeruginosa* from various clinical specimens

Source/Site	No of isolates	%
Urine	47	53.4
Wound	19	21.6
Sputum	5	5.7
Blood	5	5.7
In dwelling medical devices	12	13.6
Total	**88**	**100**

Table 3.1b: Distribution of specimen based on ward

Wards	Specimen type					
	Urine	Wound	In dwelling medical devices	Sputum	Blood	Total
Surgery	20	5	3	2	1	31
Medicine	12	2	2	2	2	20
Orthopedic	3	8	1	0	1	13
Paediatric	3	1	2	0	1	7
Neurosurgery	4	1	1	1	0	7
ICU	2	1	2	0	0	5
ENT	1	1	1	0	0	3
Gynaecology	2	0	0	0	0	2
Total	**47**	**19**	**12**	**5**	**5**	**88**

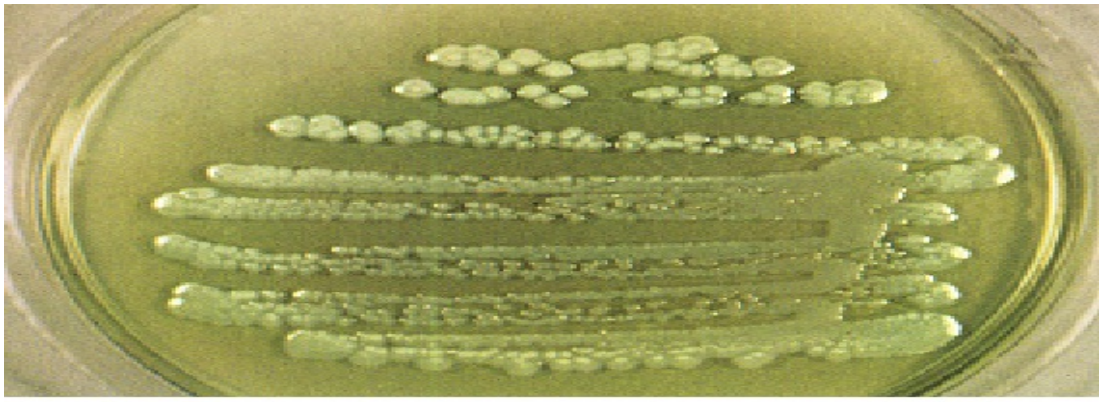


Figure 3.1a: Pyocyanin pigment formation on nutrient agar.

Figure 3.1b: Non-lactose-fermenting colonies on Mac-Conkey agar.

Figure 3.1c: Gram stain shows that bacteria are Gram–negative coccobacilli or rod

3.2 Antibiotic susceptibility test

The susceptibilities of various antibiotics against the *P. aeruginosa* clinical isolates were determined using E-test® as a quantitative antimicrobial susceptibility testing method. The susceptibility patterns of amikacin and gentamicin drugs for all 88 *P. aeruginosa* clinical isolates are shown in Figure 3.2a. Eighty-three isolates were resistant to gentamicin, 3 isolates were intermediate and only 2 isolates were sensitive to the gentamicin. Fifty-eight of the isolates were resistant to amikacin, 10 isolates were intermediate and 20 isolates were susceptible to amikacin.

The results of susceptibility testing of *P. aeruginosa* clinical isolates to ceftazidime, piperacillin/tazobactam and aztreonam are shown in Figure 3.2b. Eighty-one isolates were non-susceptible for ceftazidime, 5 isolates were intermediate, while 2 of the isolates were sensitive to ceftazidime. Interestingly, 51 of the isolates were resistant to piperacillin/tazobactam, while 6 of the isolates were intermediate for piperacillin/tazobactam, and 31 were susceptible for piperacillin/tazobactam. Although 49 *P. aeruginosa* clinical isolates were resistant to aztreonam, 27 of the isolates showed intermediate resistance, while 12 of the isolates demonstrated susceptibility to aztreonam.

The susceptibility testing of *P. aeruginosa* clinical isolates for carbapenems showed that 69 of the isolates were resistant to meropenem, while only 19 of the isolates were sensitive to meropenem. Sixty-five isolates were resistant to imipenem, while 23 of the isolates showed susceptibility to imipenem (Figure 3.2c).

Figure 3.2d shows the susceptibility of the isolates for ciprofloxacin tested by E-test®. Most of the isolates were resistant to ciprofloxacin (81), however, the intermediate resistance was only recorded for 2 isolates, while 5 isolates were sensitive to

ciprofloxacin. Most of *P. aeruginosa* clinical isolates have a high-level of susceptibility for colistin (82), while only 6 isolates were resistance to colistin (Figure 3.2d).

The activities and MIC values of the tested antipseudomonal agents towards the collection of *P. aeruginosa* clinical isolates are shown in Table 3.2a and Appendix III according to CLSI breakpoints (Tholen, 2006). The overall antibiotic resistance of clinical isolates is summarized in Figure 3.2e, which also illustrates the increased resistance level for gentamicin (94.0%), followed by ciprofloxacin and ceftazidime (92.0%), meropenem (78.0%), imipenem (74.0%), amikacin (66.0%), piperacillin/tazobactam (58.0%), aztreonam (56.0%) and colistin (7.0 %).

In the present study, the highest resistant rate of *P. aeruginosa* infections were observed in the surgical department for all antibiotics except colistin which showed the least amount of resistance followed by the medical and orthopedic departments (Table 3.2b). The MICs of different antibiotics against *P. aeruginosa* based on the site of the specimen appeared highly resistant with urine and wound specimens being most resistant to the antibiotics except for colistin (Table 3.2c).

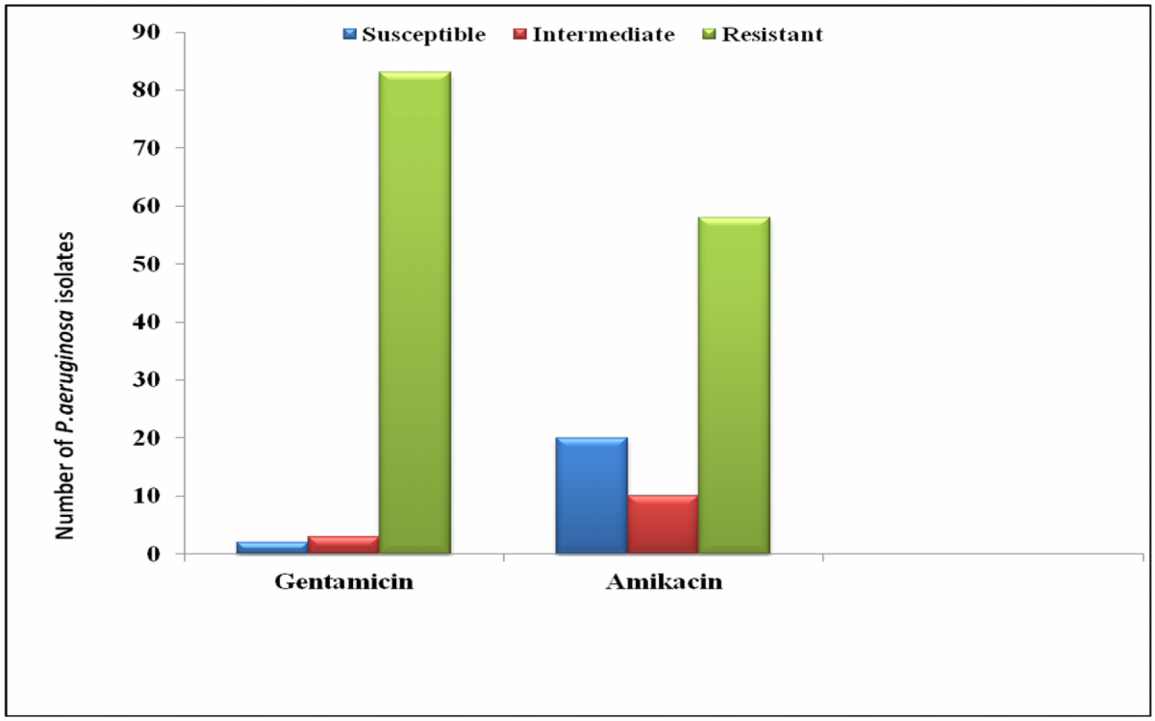


Figure 3.2 a: The susceptibility pattern of amikacin and gentamicin drugs against *P. aeruginosa* clinical isolates (n= 88).

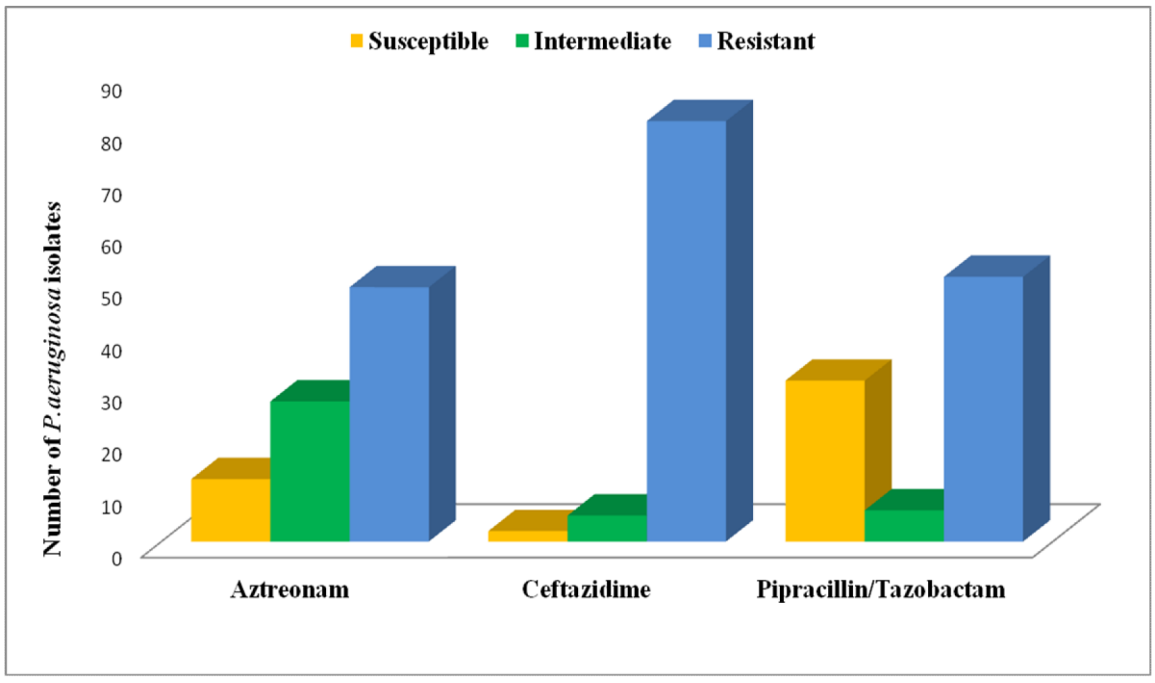


Figure 3.2b: The susceptibility patterns of aztreonam, ceftazidime and piperacillin/tazobactam drugs against *P. aeruginosa* clinical isolates (n= 88).

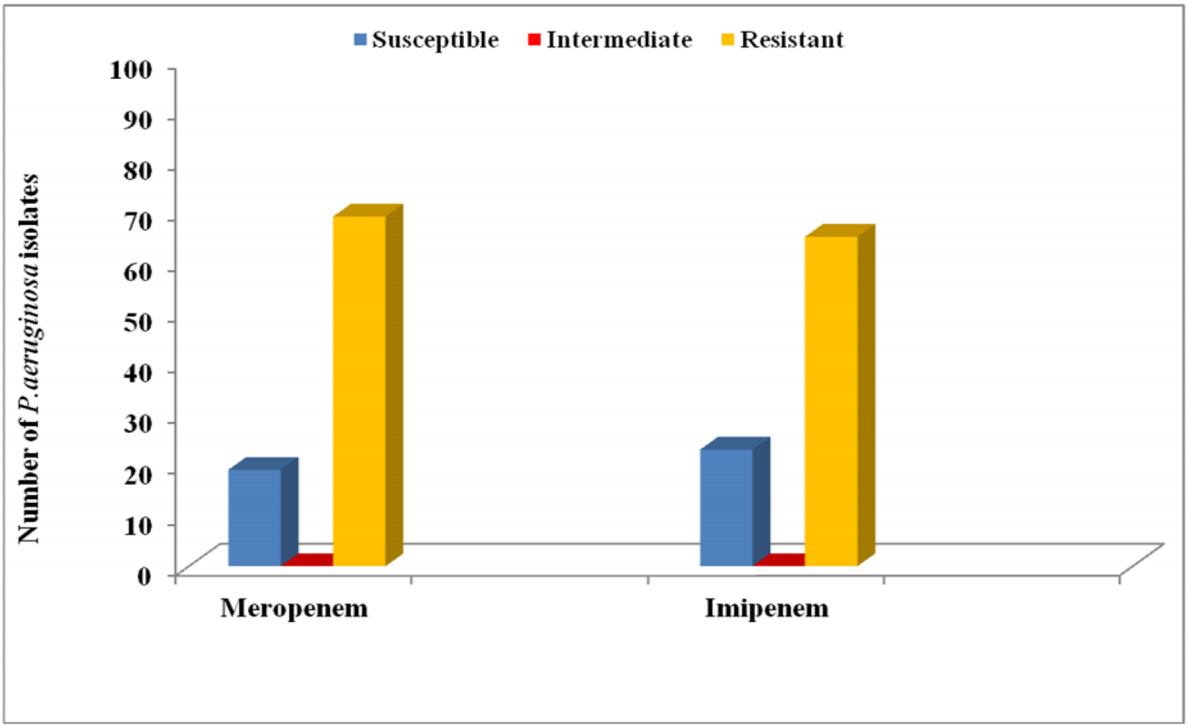


Figure 3.2c: The susceptibility distribution of imipenem and meropenem drugs against *P. aeruginosa* clinical isolates (n= 88).

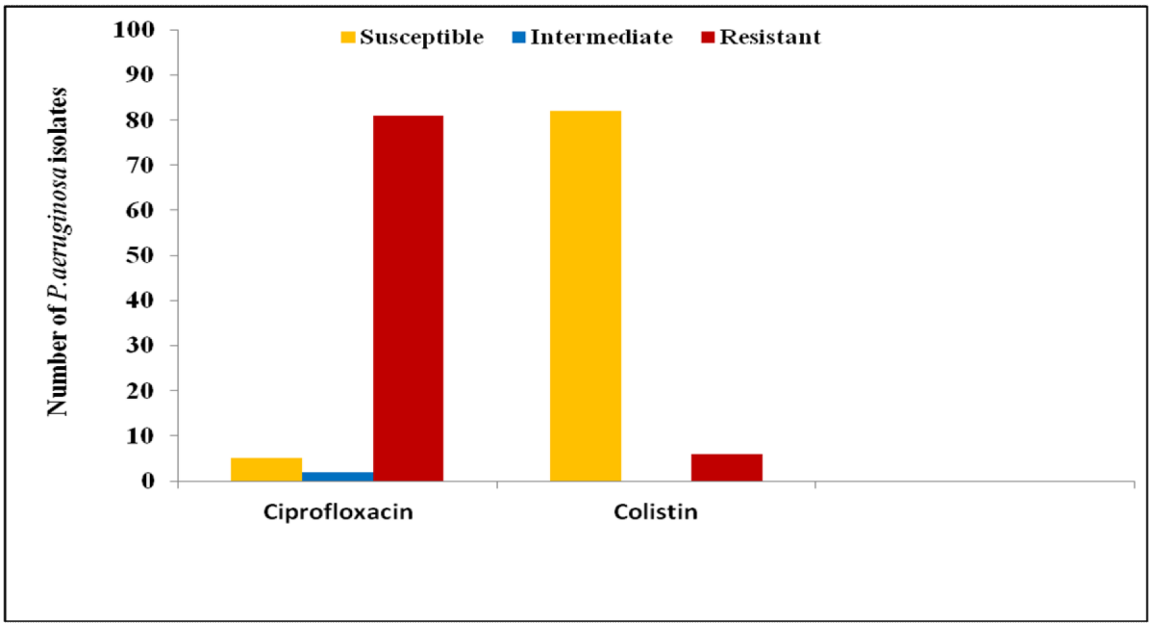


Figure 3.2d: The susceptibility distribution of ciprofloxacin and colistin drugs against *P. aeruginosa* clinical isolates (n= 88).

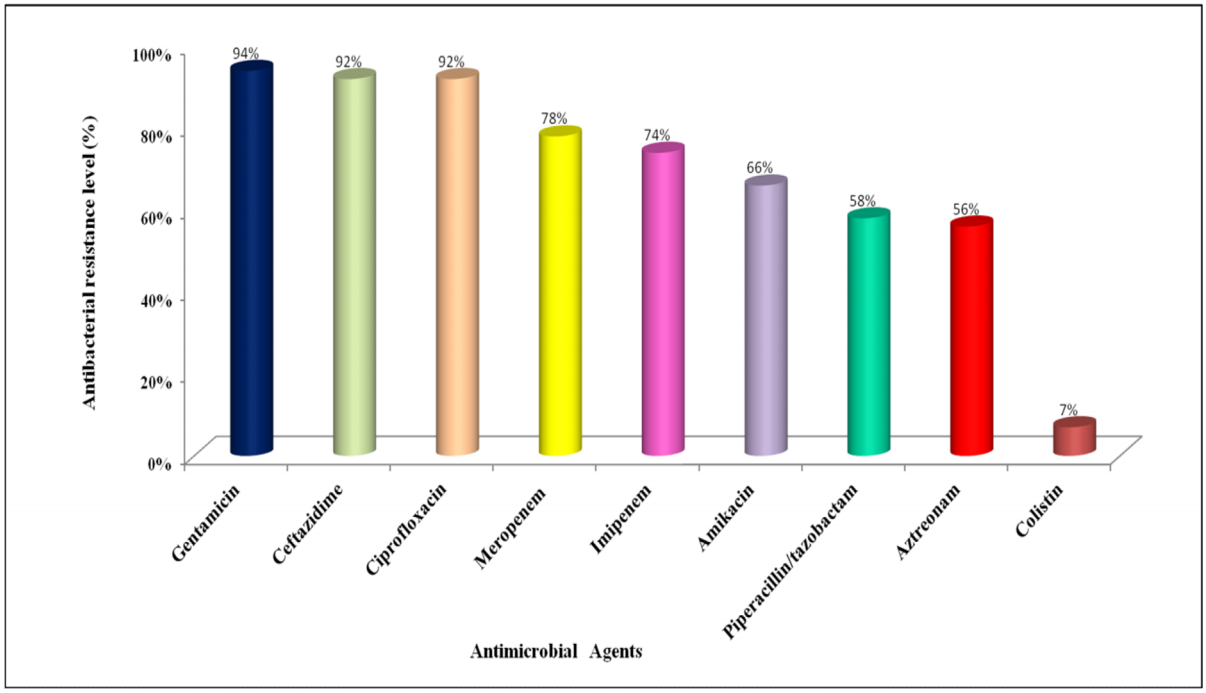


Figure 3.2e: Percentage of antibiotic resistance among of *P. aeruginosa* clinical isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88).

Table 3.2a: Activities of antipseudomonal agents toward *P. aeruginosa* clinical isolate (n=88)

Antibiotics	MIC_{50} (µg/mL)	MIC_{90} (µg/mL)	MIC range (µg/mL)	% (S) (according to CLSI)[a]	% (I) (according to CLSI)[a]	% (R) (according to CLSI)[a]
Gentamicin	2	64	0.06 –≥ 64	2	4	94
Ceftazidime	4	32	0.25–≥128	2	6	92
Ciprofloxacin	0.25	32	0.03–≥32	6	2	92
Imipenem	2	32	0.12 –≥64	26	0	74
Amikacin	4	8	0.12 –≥128	23	11	66
Piperacillin/ Tazobactam	8	128	1 –≥128	35	7	58
Aztreonam	8	32	0.25–≥128	14	30	56
Colistin	0.5	1	0.03 – 8	93	0	7

MIC: minimum of inhibition concentration, [a] percentage of isolates susceptible (S), intermediate (I), or resistant (R) according to CLSI breakpoints (Tholen, 2006).

Table 3.2b: Antibiotic resistance of *P. aeruginosa* based on ward

Antibiotics	Surgery (n= 31)	Medicine (n= 20)	Orthopedic (n= 13)	Paediatric (n= 7)	Neurosurgery (n= 7)	ICU (n= 5)	ENT (n= 3)	Gynaecology (n= 2)
Imipenem	77%	70%	69%	71%	71%	100%	33%	100%
Meropenem	84%	75%	69%	71%	85%	100%	33%	100%
Ceftazidime	100%	90%	92%	86%	100%	100%	66%	100%
Piperacillin/ Tazobactam	61%	65%	54%	100%	14%	60%	0%	50%
Aztreonam	68%	45%	54%	29%	57%	100%	0%	50%
Amikacin	45%	45%	76%	86%	14%	40%	33%	50%
Gentamicin	100%	90%	100%	86%	100%	60%	100%	100%
Ciprofloxacin	97%	90%	92%	86%	100%	60%	67%	100%
Colistin	3%	0%	8%	14%	0%	0%	0%	0%

Table 3.2c: Antibiotic susceptibility pattern of *P. aeruginosa* isolates based on site of specimen

Source	Imipenem			Meropenem			Ceftazidime			Amikacin			Aztreonam			Piperacillin /tazobactam			Ciprofloxacin			Gentamicin			Colistin		
	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R	S	I	R
Urine	13	0	34	10	0	37	1	0	46	10	17	20	3	12	32	19	3	25	0	0	47	1	0	46	45	1	1
Wound	6	0	13	5	0	14	0	1	18	5	4	11	5	8	8	6	1	12	1	2	16	0	1	18	16	3	0
Sputum	0	0	5	0	0	5	1	0	4	2	0	3	1	0	4	1	1	3	1	0	4	0	1	4	5	0	0
Blood	2	0	3	2	0	3	0	0	5	0	0	5	0	4	1	1	0	4	0	0	5	0	0	5	5	0	0
In dwelling*	2	0	10	2	0	10	2	0	10	3	3	6	3	3	6	4	1	7	1	3	8	1	1	10	10	0	2
Total	23	0	65	19	0	69	4	1	83	20	24	45	12	27	51	31	6	51	3	5	80	2	3	83	81	4	3

** In dwelling medical devices, S (susceptible), I (intermediate) and R (resistant)*

3.3 Molecular analysis of antibiotic patterns and gene regulation results

The current study aimed to determine the gene expression profiles of the Malaysian isolates of *P. aeruginosa* using real time PCR to evaluate the resistance mechanisms responsible for antibiotic resistances among the *P. aeruginosa* clinical isolates. The gene expression levels of ampC cephalosporinase, the multidrug efflux pumps, the OprD outer membrane porin and two penicillin binding proteins (PBP2, PBP3) were investigated and the antibiotic susceptibility patterns of these isolates were determined in association with the mRNA gene expression in comparison to *P. aeruginosa* ATCC 27583.

3.3.1 Aminoglycosides

Twenty isolates were susceptible to amikacin according to the CLSI breakpoints (Table 3.3.a). Among these isolates, the *mexB* gene was upregulated in 15 isolates, 14 isolates for both *mexZ* and *ampC*, 12 isolate showed upr-egulated *mexY and mexEF* and only 10 isolates showed *mexCD* gene up-regulation. Also, the up-regulation of *oprM*, *PBP2, PBP3* and *oprD* was noted in 8, 6, 4 and 2 of the isolates, respectively. In contrast, among these isolates, down-regulation was found in 5 isolates with the (*mexB*), in 6 isolates (both *mexZ* and *ampC*) and in 8 isolates (both *mexY* and *mexEF*). For the *oprD , PBP3, PBP2* and *oprM* genes*,* down-regulation was noted in the 18, 16, 14 and 12 of the susceptible isolates, respectively, in comparison to *P. aeruginosa* ATCC 27583.

Ten isolates had intermediate susceptibility to amikacin based on the CLSI breakpoints (Table 3.3.a). Amongst these, the up-regulation of *mexEF*, *ampC*, *mexY*, *mexB*, *mexCD* and *mexZ* genes was noted in 9, 8, 8, 7, 5 and 4 isolates, respectively. However, in these ten isolates, *oprD, PBP2* and *PBP3* were down-regulated, while in nine of isolates only *oprM* was down-regulated. A total of 58 isolates were found to be resistant to amikacin. The *mexY* gene was upregulated in 52 isolates. For the others, *mexB*, *mexZ*, *mexEF*, *ampC* and *mexCD* up-regulation was noted in 42, 39, 39, 35 and 27 of isolates

respectively. Down-regulation was noted for *oprD* in 57 of the isolates. Also, *PBP3*, *PBP2* and *oprM* gene expression was down-regulated in 48, 46 and 36 of these isolates respectively (Table 3.3.a).

For gentamicin, only 2 isolates were susceptible (Table 3.3.a), among these isolates, up-regulation was noted only with 1 isolate for the *ampC*, *mexEF*, *PBP2* and *PBP3* genes. However, among these two isolates down-regulation was noted for *mexB*, *mexZ*, *mexY*, *mexCD, oprD, PBP2* and *oprM* but not for *ampC*, *mexEF*, and *PBP3* which was seen only in one isolate (Table 3.3.a). With the 3 isolates that had intermediate resistance to gentamicin, up-regulation of *mexB*, *mexZ* and *mexEF* was found in 2 of these isolates and in 1 isolate for *ampC*, *mexY* and *mexCD*. In all of the 3 isolates the *oprD, PBP2* and *oprM* genes, were down-regulated while in 2 isolates *ampC*, *mexY*, *mexCD* and *PBP3* were also found down-regulated.

Eighty-three of the isolates were found to be resistant to gentamicin, of which 71 had up-regulation of *mexY* gene, followed by 62 for *mexB,* 57 for *mexEF* gene and 55 for both *mexZ* and *ampC*. Down-regulation was noted in 80 isolates for *oprD* , while, for genes *PBP3*, *PBP2* and *oprM,* 71, 64 and 52 were down-regulated in these isolates as compared to *P. aeruginosa* ATCC 27853 (Table 3.3.a).

Table 3.3.a: Distribution of mRNA gene expression of aminoglycoside resistant isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88)

Antibiotics		*mexB*			*mexZ*			*mexY*			*ampC*			*mexCD*			*mexEF*			*oprD*			*PBP2*			*PBP3*			*OprM*		
		↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T
Amikacin	S	15	5	20	14	6	20	12	8	20	14	6	20	10	10	20	12	8	20	2	18	20	6	14	20	4	16	20	8	12	20
	I	7	3	10	4	6	10	8	2	10	8	2	10	5	5	10	9	1	10	0	10	10	2	8	10	0	10	10	1	9	10
	R	42	16	58	39	19	58	52	6	58	35	23	58	27	31	58	39	19	58	1	57	58	12	46	58	10	48	58	22	36	58
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88
Gentamicin	S	0	2	2	0	2	2	0	2	2	1	1	2	0	2	2	1	1	2	0	2	2	1	1	2	1	1	2	0	2	2
	I	2	1	3	2	1	3	1	2	3	1	2	3	1	2	3	2	1	3	0	3	3	0	3	3	1	2	3	0	3	3
	R	62	21	83	55	28	83	71	12	83	55	28	83	41	42	83	57	26	83	3	80	83	19	64	83	12	71	83	31	52	83
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88

S (susceptible), I (intermediate) and R (resistant), T (Total), ↑ = up-regulation and and ↓= down-regulation.

3.3.2 β-lactamase drugs

Two isolates were found to be susceptible to ceftazidime, and these isolated demonstrated up-regulation of *mexZ, mexY, ampC, mexCD* and *mexEF* gene expression. Also these 2 isolates showed down-regulation of *oprM* expression. However, one of the isolates had down-regulation for *oprD*, *PBP2* and *PBP3* expression as shown in Table 3.3b. Five of the isolates showed intermediate susceptibility for ceftazidime and 4 of them showed up-regulation of *mexY, mexEF* and *ampC,* although *oprD* was down-regulated in all as shown in Table 3.3b.

Eighty-one of the isolates were found to be resistant to ceftazidime. Sixty-six of these isolates showed up-regulation of *mexY,* 60 of *mexB* and 54 of *mexEF*. On the other hand, 79, 69, 63 and 52 of these isolates demonstrated down-regulation for *oprD, PBP3*, *PBP2* and *oprM* gene expression respectively (Table 3.3b).

Thirty-one of the isolates were found to be susceptible to piperacillin/tazobactam. Up-regulation of (*mexY*, *mexEF*, *mexB* and *mexZ*) gene expression was noted in 25, 23, 22 and 18 of these isolates, respectively. In addition, 15 isolates showed up-regulation of both *mexCD* and *ampC* gene expression. On the contrary, all of the 31 isolates demonstrated down-regulation of *oprD* followed by 28, 26 and 20 for *PBP3, PBP2* and *oprM* gene, respectively (Table 3.3b).

Six of the isolates had intermediate susceptibility for piperacillin/tazobactam and 5 of these isolates had up-regulation of *mexB, mexZ* and *mexEF* expression while for *ampC* and *mexCD,* up-regulation was noted in only 4 isolates. However, down-regulation was noted with all of these 6 isolates for *oprD* and in 4, 3, 3 of these isolates for *PBP2, PBP3* and *oprM* gene, respectively, as shown in Table 3.3b.

Fifty-one of the isolates were resistant to piperacillin/tazobactam, while 44, 38, 37 and 32 of these isolates showed up-regulation of *mexY, ampC, mexB* and *mexEF* expression

respectively. However, down-regulation of *oprD* *PBP3, PBP2* and *oprM* gene expression was noted in 48, 43, 38 and 34 of these isolates, respectively (Table 3.3b).

Twelve of the isolates were sensitive to aztreonam. Up-regulation of expression was noted with 11 of these isolates for *mexY,* in 8 isolates for both *mexEF* and *mexB* and 7 isolates for both *mexZ* and *ampC*. On the other hand, down-regulation of expression was noted with all of these 12 isolates for *oprD*, also in 10, 9 and 8 of these isolates for *PBP3, PBP2* and *oprM,* respectively (Table 3.3b).

Twenty-seven of the isolates demonstrated an intermediate susceptibility for aztreonam. Twenty-four of these isolates showed up-regulation of *mexY,* followed by 20 of these isolates of both *mexB* and *mexEF*. However, down-regulation was noted in 25 of these isolates for *oprD* and in 22 of these isolates for both *PBP2* and *PBP3* (Table 3.3b).

Forty-nine of the isolates were found to be resistant to aztreonam. Up-regulation was noted in 37, 36, 33 and 32 of these isolates for *mexY*, *mexB, mexZ* and *mexEF* respectively. Whereas, down-regulation was noted in 48, 42 and 37 of these isolates for *oprD, PBP3* and *PBP2* respectively (Table 3.3b).

For carbapenem drugs, 19 isolates were found to be sensitive for meropenem and 17 of these isolates had up-regulated for *mexY* as well as up-regulated both *mexB* and *mexZ* that was found in 15 of the isolates. Sixteen of these isolates showed down-regulated *oprD* expression as illustrated in Table 3.3c.

Sixty-nine of the isolates showed resistance to meropenem. Up-regulation was noted in 55, 49, 48 and 45 of these isolates for *mexY*, *mexB, mexEF* and *ampC*, in that order. Conversely, all of the 69 isolates had down-regulated *oprD*, except for *PBP3* and *PBP2,* which were found to be down-regulated in 62 and 55 correspondingly (Table 3.3c).

In regards to carbapenem drugs, 23 of the isolates showed susceptibility for imipenem and 20 of these isolates had up-regulation *mexY* expression followed by 18 isolates that showed up-regulated both *mexB* and *mexZ* expression. On the other hand, down-

regulation was noted in 20, 16 and 15 of these isolates for *oprD, PBP2* and *PBP3* expression, respectively (Table 3.3c).

Sixty-five of the isolates were found to be resistant to imipenem. Up-regulation was noted in 52, 46, 44, and 43 of these isolates for *mexY*, *mexB, mexEF* and *ampC* respectively. However, down-regulation was observed in all 65 of the isolates for *oprD* as well as in 59, 52 and 43 for *PBP3*, *PBP2* and *oprM* respectively, as illustrated in Table 3.3c.

Table 3.3b: Distribution of mRNA gene expression of β-lactamase resistant isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88)

Antibiotics		*mexB*			*mexZ*			*mexY*			*ampC*			*mexCD*			*mexEF*			*oprD*			*PBP2*			*PBP3*			*oprM*		
		↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T
Ceftazidime	S	1	1	2	2	0	2	2	0	2	2	0	2	2	0	2	2	0	2	1	1	2	1	1	2	1	1	2	0	2	2
	I	3	2	5	2	3	5	4	1	5	4	1	5	1	4	5	4	1	5	0	5	5	1	4	5	1	4	5	2	3	5
	R	60	21	81	53	28	81	66	15	81	51	30	81	39	42	81	54	27	81	2	79	81	18	63	81	12	69	81	29	52	81
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88
Piperacillin/ Tazobactam	S	22	9	31	18	13	31	25	6	31	15	16	31	15	16	31	23	8	31	0	31	31	5	26	31	3	28	31	11	20	31
	I	5	1	6	5	1	6	3	3	6	4	2	6	2	4	6	5	1	6	0	6	6	2	4	6	3	3	6	3	3	6
	R	37	14	51	34	17	51	44	7	51	38	13	51	25	26	51	32	19	51	3	48	51	13	38	51	8	43	51	17	34	51
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88
Aztreonam	S	8	4	12	7	5	12	11	1	12	7	5	12	6	6	12	8	4	12	0	12	12	3	9	12	2	10	12	4	8	12
	I	20	7	27	17	10	27	24	3	27	19	8	27	9	18	27	20	7	27	2	25	27	5	22	27	5	22	27	10	17	27
	R	36	13	49	33	16	49	37	12	49	31	18	49	27	22	49	32	17	49	1	48	49	12	37	49	7	42	49	17	32	49
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88

S (susceptible), I (intermediate) and R (resistant), T (Total), ↑ = up-regulation and ↓= down-regulation

Table 3.3c: Distribution of mRNA gene expression of carbapenem resistant isolates

Antibiotics		*mexB*			*mexZ*			*mexY*			*ampC*			*mexCD*			*mexEF*		
		↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	
Meropenem	S	15	4	19	15	4	19	17	2	19	7	12	19	4	15	19	12	7	
	R	49	20	69	42	27	69	55	14	69	45	24	69	38	31	69	48	21	6
	T	69	24	88	57	31	88	72	16	88	52	26	88	42	46	88	60	28	8
Imipenem	S	18	5	23	18	5	23	20	3	23	14	9	23	7	16	23	16	7	2
	R	46	19	65	39	26	65	52	13	65	43	22	65	35	30	65	44	21	6
	T	64	24	88	57	31	88	62	16	88	57	31	88	42	46	88	60	28	8

in comparison to *P. aeruginosa* ATCC 27583 (n=88)

S (susceptible), I (intermediate) and R (resistant), T (Total), ↑ = up-regulation and ↓= down-regulation.

3.3.3 Fluoroquinolones

Five of the isolates were susceptible to ciprofloxacin. Up-regulation was noted in 4 of these isolates for *mexEF* and in 3 of them for *mexZ, ampC* and *mexCD* genes. However, down-regulation was evident in all of these five isolates for *oprD* as well as in 4, 3, and 3 of these isolates for *oprM*, *PBP2* and *PBP3,* respectively (Table 3.3d).

Two of the isolates were intermediately susceptible to ciprofloxacin. Up-regulation was noted in two isolates for *mexY and mexCD* and only in one isolate for *mexB* and *mexEF*. On the other hand, both these isolates had down-regulation *oprD, PBP2, PBP3* and *oprM* expression (Table 3.3d).

Eighty-one of the isolates were resistant to ciprofloxacin. Up-regulation was evident in 68, 61 and 55 of these isolates for *mexY, mexB* and *mexEF,* respectively, as well as in 53 of these isolates for both *mexZ* and *ampC*. However, down-regulation was noted in 78, 69 and 63 of these isolates for *oprD, PBP3* and *PBP2* in that order (Table 3.3d).

3.3.4 Polymyxin E (Colistin)

Eighty-two of the isolates were found to be susceptible to colistin. Up-regulation was noted in 68, 60 and 55 of these isolates for *mexY, mexB* and *mexEF,* respectively, whereas, down-regulation was observed in 79, 71 and 65 of these isolates for *oprD, PBP3* and *PBP2,* respectively (Table 3.3d).

Six of the isolates were resistant to colistin and all of these isolates had up-regulation for *mexB, mexY, ampC* and *mexEF* gene expression. However, down-regulation observed in all of these isolates for *oprD* and *oprM* genes (Table 3.3d).

Table 3.3d: Distribution of mRNA gene expression in ciprofloxacin and colistin resistant isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88)

Antibiotics		*mexB*			*mexZ*			*mexY*			*ampC*			*mexCD*			*mexEF*			*oprD*			*PBP2*			*PBP3*			*oprM*		
		↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T	↑	↓	T
Ciprofloxacin	S	2	3	5	3	2	5	2	3	5	3	2	5	3	2	5	4	1	5	0	5	5	2	3	5	2	3	5	1	4	5
	I	1	1	2	1	1	2	2	0	2	1	1	2	0	2	2	1	1	2	0	2	2	0	2	2	0	2	2	0	2	2
	R	61	20	81	53	28	81	68	13	81	53	28	81	39	42	81	55	26	81	3	78	81	18	63	81	12	69	81	30	51	81
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88
Colistin	S	60	22	82	52	30	82	68	14	82	54	28	82	40	42	82	55	27	82	3	79	82	17	65	82	11	71	82	30	52	82
	I	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	R	6	0	6	6	0	6	6	0	6	6	0	6	2	4	6	6	0	6	0	6	6	3	3	6	3	3	6	0	6	6
	T	64	24	88	57	31	88	72	16	88	57	31	88	42	46	88	60	28	88	3	85	88	20	68	88	14	74	88	31	57	88

S (susceptible), I (intermediate) and R (resistant), T (Total), ↑ = up-regulation and ↓= down-regulation.

3.3.5 Overall gene expression analysis of chromosomal resistance genes

The gene expression analysis of 88 *P. aeruginosa* isolates showed overexpression of efflux pump genes for *mexY* (82.0%, from 2.0 to 1731.0 fold), *mexB* (73.0%, from 2.0 to 50.0 fold), *mexEF* (68.0%, from 2.9 to 371), *mexZ* (66.0%) and *mexCD* (48.0%, from 2.0 to 522), while for *ampC* overexpression it was 65.0% (from 10.4 to 1806.0 fold). Down-regulation were noted for *oprD* (97.0%, from 0.2 to 0.7 fold), *PBP2* (77.0%, from 0.1 to 0.7 fold), *PBP3* (84.0%, from 0.01 to 0.7 fold) and *oprM* (65%, from 0.2 to 0.7) as compared to expression of the respective genes in *P. aeruginosa* ATCC 27853 (Figure 3.3 and Appendix III).

Among the resistant isolates overexpressing *mexB*, the lowest mRNA expression was noted for isolates resistant to colistin (67%), whereas the highest mRNA expression was noted for isolates resistant to ciprofloxacin (78%). In relation to isolates that were resistant to meropenem, imipenem, gentamicin, ceftazidime, amikacin, aztreonam and piperacillin/tazobactam, the range of *mexB* mRNA expression ranged from 71% to 77% (Table 3.3e).

When *mexY* gene overexpression was examined, the lowest and highest mRNA expression were seen for isolates resistant to colistin (67%) and amikacin (90%) , whilst the mRNA expression of this gene for isolates resistant to gentamicin, ciprofloxacin, ceftazidime, aztreonam and piperacillin/tazobactam ranged from 76% to 86% as depicted in Table 3.3e.

For the overexpression of *mexCD*, the lowest mRNA expression was seen with isolates resistant to gentamicin and piperacillin/tazobactam (49%). However, the highest overexpression was seen with isolates resistant to meropenem and aztreonam (55%). In relation to isolates resistant to other antibiotics, *mexCD* mRNA expression (up-regulation) ranged from 47% to 54% (Table 3.3e).

For the overexpression of *mexEF*, the lowest mRNA expression was observed with isolates resistant to piperacillin/tazobactam (63%), and the highest mRNA expression was noted with isolates resistant to colistin (100 %). In relation to the isolates that were resistant to other antibiotics, mRNA expression of these isolates for *mexEF* gene up-regulation ranged from 65% to 70% (Table 3.3e).

For the overexpression of *ampC* gene, the lowest mRNA expression was seen with isolates resistant to colistin (50%) and the highest mRNA expression was noted with isolates resistant to piperacillin/tazobactam (75%). In relation to the resistant isolates to other antibiotics, mRNA expression of these isolates for *ampC* gene up-regulation ranged from 60% to 66.0% (Table 3.3e).

The *oprD*, mRNA expression of isolates resistant to meropenem, imipenem and colistin demonstrated 100% down-regulation and in relation to the resistant isolates to other antibiotics, mRNA expression of these isolates for *OprD* gene down-regulation ranged from 94% to 98% (Table 3.3d).

For *oprM,* the isolates resistant to amikacin were noted to have the lowest mRNA expression (62%), while the highest mRNA expression was seen with isolates resistant to colistin that showed 100% down-regulation for *oprM* gene. In relation to isolates resistant to other antibiotics, mRNA expression of these isolates for *oprM* shows that down-regulation ranged from 63% to 67% (Table 3.3e).

For *PBP2* gene*,* isolates resistant to colistin had the lowest mRNA expression (50%), while the highest mRNA expression value was 80% for isolates resistant to meropenem and imipenem. On the other hand, for the *PBP3* gene, the mRNA expression was down-regulated and showed the lowest and highest mRNA expression for isolates resistant to colistin (50%) and imipenem (91%) respectively. However, in relation to the resistant isolates to other antibiotics investigated, these isolates showed low mRNA expression for *PBP2* and *PBP3* which varied from 75% to 86% (Table 3.3e).

With regard to the significance of the above results, the overexpression patterns of *mexY* were significantly different in isolates resistant to amikacin (90%), gentamicin (86%) and ciprofloxacin (84%) as compared to other antibiotics ($p < 0.05$). On the other hand, for *ampC*, mRNA expression in *P. aeruginosa* isolates demonstrated high significant differences towards piperacillin/tazobactam antibiotics as compared to other antibiotics ($p < 0.05$).

All *P. aeruginosa* clinical isolates that were resistant to imipenem and meropenem showed significantly decreased *oprD* expression ($p = 0.016$). The down-regulation of mRNA expression of *PBP3* was observed in isolates resistant to meropenem (90%) and imipenem (91%) ($p = 0.04$). Likewise, the down-regulation of penicillin binding protein (*PBP3*) demonstrated high significant in isolates that were resistance to piperacillin/tazobactam (84%) ($p = 0.047$) as compared to *P. aeruginosa* ATCC 27853 (Table 3.3e and Table 3.3f).

Of 88 isolates, 85 had 100% down-regulation of *oprD* gene. These 85 isolates were further analyzed and the results are shown in Table 3.3g. The analysis of the efflux pump overexpression gene (mex^+) showed the lowest mRNA expression in isolates that were resistant to colistin (50%) and the highest mRNA expression was seen in isolates resistant to meropenem (65%). On the other hand, in relation to isolates resistant to other antibiotics, mRNA expression of these isolates for mex^+ were up-regulated (range from 52% to 55%) (Table 3.3g). In those isolates, *ampC* showed the lowest and highest mRNA expressions in isolates that were resistant to colistin (50%) and piperacillin/tazobactam (69%), respectively. In addition, the mRNA expression in these isolates for *PBP* genes demonstrated down-regulation in isolates resistant to imipenem (75%) and colistin (50%) showing the lowest mRNA expression. Furthermore, the *oprM* mRNA expression level in these isolates was down-regulated in isolates that were

resistant to meropenem and imipenem (67%) and the highest mRNA expression was seen in isolates resistant to colistin (Table 3.3g).

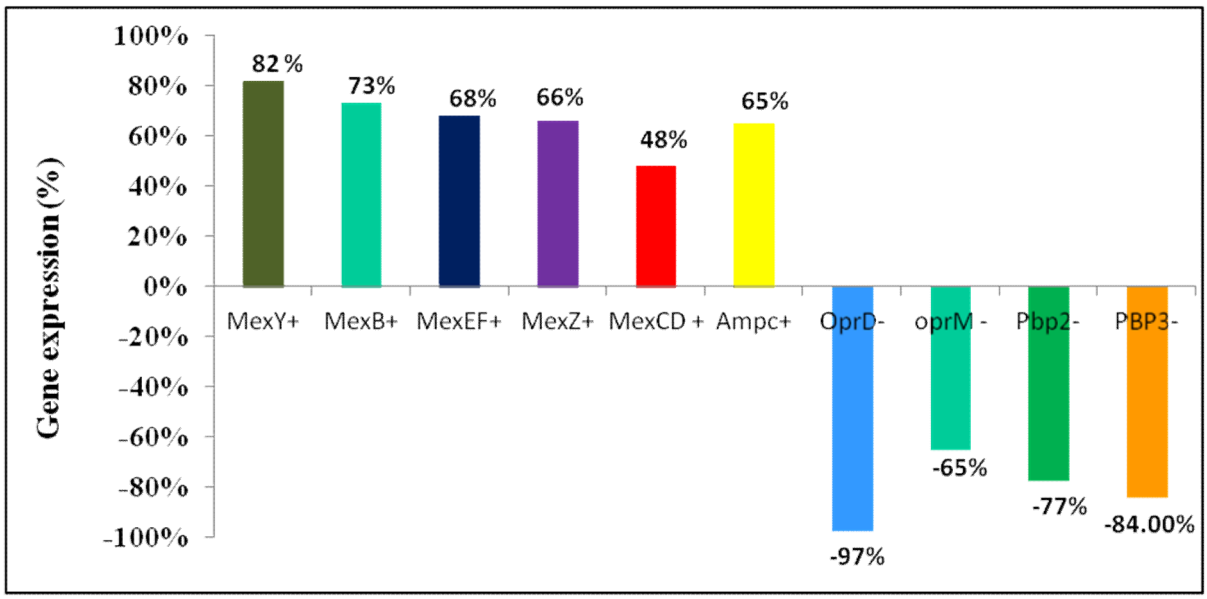


Figure 3.3: The percentage change of gene expression levels of *P. aeruginosa* clinical isolate in comparison to *P. aeruginosa* ATCC 27583 (n=88).

Table 3.3e: Percentage of mRNA transcription levels of gene expression of multidrug resistant isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88)

Resistance of Antibiotics	mRNA expression for:									
	Efflux pump					β-Lactamase	Out membrane protein		Penicillin binding Protein	
	mexB^{+}	*mexZ*$^{+}$	*mexY*$^{+}$	*mexCD*$^{+}$	*mexEF*$^{+}$	*ampC*$^{+}$	*oprD*$^{-}$	*oprM*$^{-}$	*PBP2*$^{-}$	*PBP3*$^{-}$
Gentamicin	77.0	68.0	86.0*	49.0	69.0	66.0	97.0	63.0	77.0	86.0
Ceftazidime	77.0	67.0	82.0	48.0	67.0	63.0	98.0*	64.0	78.0	85.0
Ciprofloxacin	78.0	67.0	84.0*	48.0	68.0	65.0	96.0	63.0	78.0	85.0
Meropenem	71.0	61.0	80.0	55.0*	70.0	65.0	100*	67.0	80.0*	90.0*
Imipenem	74.0	62.0	80.0	54.0*	68.0	66.0	100*	66.0	80.0	91.0*
Amikacin	72.0	67.0	90.0*	47.0	67.0	60.0	98.0	62.0	79.0	83.0
Piperacillin/ Tazobactam	77.0	69.0	86.0	49.0	63.0	75.0*	94.0	67.0	75.0	84.0*
Aztreonam	74.0	67.0	76.0	55.0	65.0	63.0	98.0	65.0	76.0	86.0
Colistin	67.0	83.0	67.0	50.0	100	50.0	100	100	50.0	50.0

All values are in percentage (%)
*Significantly different from other groups (p <.05).
The abbreviations mexB^{+}, mexY^{+} , mexCD^{+}, mexEF^{+} and ampC^{+} designate mexAB-OprM, mexXY, mexCD, mexEF and ampC overexpression, respectively.
oprD^{-}: oprD porin down-regulation, oprM^{-} : oprM out membrane protein down regulation.
PBP2^{-} and PBP3^{-} penicillin binding protein down-regulation.

Table 3.1f Associations of susceptibility patterns of different antibiotics toward *P. aeruginosa* clinical isolates with different gene expressions

Antibiotics	GENE	S. patterns [a]		UP	DOWN	chi-square	*p*-value
Gentamicin	*mexY*	S	5	1	4	14.5	0.001
		R	83	71	12		
Ceftazidime	*oprD*	S	7	1	6	13.5	0.001
		R	81	2	79		
Ciprofloxacin	*mexY*	S	7	4	3	6.5	0.037
		R	81	68	13		
Imipenem	*oprD*	S	23	3	20	8.7	0.016*
		R	65	0	65		
	PBP3	S	23	7	16	4.8	0.04*
		R	65	7	58		
Amikacin	*mexY*	S	30	20	10	8.8	0.012
		R	58	52	6		
Piperacillin/ Tazobactam	*ampC*	S	37	19	18	5.7	0.05
		R	51	38	13		
	PBP3	S	37	6	31	6.1	0.047
		R	51	8	43		

* *Fisher's exact test is to be used if less than 50% of the cells are less than 5.*
[a] *S. pattern: susceptibility patterns of antibiotics.*
Up: up- regulation, down -regulation
S: susceptible or R: resistant

Table 3.3g: Percentage expression level of resistance genes according to the mechanism of resistance and its association to *OprD* down-regulation according to mechanism of resistant in comparison to *P. aeruginosa* ATCC 27583 (n=85)

Resistance of Antimicrobial	mRNA expression for:			
	Efflux pump	β-Lactamase	Penicillin binding Protein	Out membrane Protein
	mex^{+}	*ampC*$^{+}$	*PBP*$^{-}$	*oprM-*
Gentamicin	54.0	63.0	70.0	64.0
Ceftazidime	53.0	61.0	70.0	66.0
Ciprofloxacin	53.0	62.0	70.0	64.0
Meropenem	65.0	65.0	74	67.0
Imipenem	53.0	66.0	75.0	67.0
Amikacin	53.0	59.0	72.0	63.0
Piperacillin/Tazobactam	52.0	69.0	67.0	69.0
Aztreonam	55.0	61.0	71.0	67.0
Colistin	50.0	50.0	50.0	100

All values are in percentage (%)
The abbreviations Mex^{+} and ampC^{+} designate mexAB-OprM, mexXY-oprM, mexCD, mexEF and ampC overexpression, respectively.
PBP^{-} : penicillin binding protein (PBP2 and PBP3) down-regulation, OprM^{-} : oprM out membrane protein down regulation.

3.4 PCR amplification of beta-lactamases genes

Sixty-five of the clinical *P. aeruginosa* isolates that were resistant to imipenem and meropenem were evaluated for presence of six different metallo-beta-lactamase (MBL) genes (*bla*$_{IMP}$, b*la*$_{VIM}$, *bla*$_{GIM}$, *bla*$_{SIM}$, *bla*$_{SPM}$ and *bla*$_{NDM}$). In addition, these isolates were tested for presence of extended spectrum beta-lactamase (ESBL) production genes (*bla*$_{VEB}$, *bla*$_{TEM}$, *bla*$_{CTX-M}$, *bla*$_{SHV}$ and *bla*$_{PER}$) using PCR (Table 3.4a). Of these isolates, 35 were from urine, 13 from wounds, 10 from indwelling medical devices, 4 from sputum and 3 from blood (Table 3.4b). Samples were collected from patients hospitalized in the surgical ward (n = 23), medical ward (n = 15), orthopedic ward (n = 9), paediatric ward (n = 5), neurosurgery ward (n = 5), intensive care unit (n = 5), gynaecology ward (n = 2) and otorhinolaryngology ward (n = 1) as shown in Table 3.4c.

Thirty-three out of 65 of the imipenem and meropenem resistant *P. aeruginosa* isolates used in the study tested were positive for ESBL genes. Out of 33 ESBL, genes were amplified in 25 isolates for *bla*$_{VEB}$, in 5 isolates for *bla*$_{TEM}$ and in 3 isolates for *bla*$_{CTX-M}$. No *bla*$_{SHV}$ and *bla*$_{PER}$ genes were detected for imipenem and meropenem resistant isolated in this study as illustrated in Table 3.4a and gel picture (Figure 3.4). All ESBL positive isolates were multi-resistant to ceftazidime, piperacillin/tazobactam, aztreonam, gentamicin and ciprofloxacin. However, when colistin was tested, all ESBL positive isolates in this study were susceptible to this antibiotic (Table 3.4d). Twenty-three of the 35 isolates from urine specimens tested positive for *bla*$_{VEB,}$ *bla*$_{TEM}$ and *bla*$_{CTX-M}$ genes and only 10 isolates from other clinical samples tested positive for the same genes (Table 3.4b). As shown in Table 3.4c, 16 of the 23 isolates from surgical wards, 5 of the 15 isolates from the medical wards and 5 of the 9 isolates from orthopedic wards tested positive for all above genes. Only seven isolates from other wards tested positive for the above genes.

Among the 65 isolates of *P. aeruginosa* resistant to imipenem and meropenem, 41 isolates were MBL producers. Out of 41, MBL, genes were amplified in 20 isolates for bla_{IMP}, in 14 isolates for bla_{VIM}, in 4 isolates for bla_{GIM}, in 2 isolates for bla_{NDM} and only in one isolate for bla_{SIM} as shown in Table 3.4a and gel picture (Figure 3.4). No bla_{SPM} gene was amplified in this isolates. The antibiotic resistance rates were higher for MBL producing isolates than for non-MBL-producing *P. aeruginosa* isolates (Table 3.4d). All MBL positive isolates were resistant to ceftazidime and all of these isolates were 100% sensitive to colistin (Table 3.4d). Most of the resistant isolates harboring MBL genes were collected from urine and wound specimens (Table 3.4b). As seen in Table 3.4c, 20 of the 23 isolates from the surgical ward, eight of the 15 isolates from the medical ward and six of the nine isolates from the orthopedic ward were amplified for bla_{IMP}, bla_{VIM}, bla_{GIM}, bla_{NDM} and bla_{SIM} genes. Only seven isolates from other wards tested positive for these genes.

Table 3.4a: Prevalence of ESBL and MBL genes among *P. aeruginosa* isolates (n = 65)

Class	Type of genes	Positive No.
ESBL gene positive (n = 33)	bla_{VEB}	25
	bla_{TEM}	5
	bla_{CTX-M}	3
MBL gene positive (n = 41)	bla_{IMP}	20
	bla_{VIM}	14
	bla_{GIM}	4
	bla_{NDM}	2
	bla_{SIM}	1

Table 3.4b: The distribution of *P. aeruginosa* isolated from various clinical specimens (n= 65)

specimens		Gene types							
		bla_{IMP}	bla_{VIM}	bla_{NDM}	bla_{SIM}	bla_{GIM}	bla_{TEM}	bla_{CTX-M}	bla_{VEB}
	No.	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**	**+ve**
Urine	35	10	9	1	0	1	3	2	18
Wound	13	5	3	0	0	1	1	0	3
In dwelling medical devices	10	2	1	0	0	0	0	0	4
Sputum	4	1	0	1	1	0	0	1	0
Blood	3	2	1	0	0	1	1	0	0
Total	**65**	**20**	**14**	**2**	**1**	**3**	**5**	**3**	**25**

Table 3.4c: The distribution of *P. aeruginosa* from different wards (n= 65)

Wards	Gene types								
	No.	bla_{IMP}	bla_{VIM}	bla_{NDM}	bla_{SIM}	bla_{GIM}	bla_{TEM}	bla_{CTX-M}	bla_{VEB}
Surgery	23	12	6	0	0	2	1	2	13
Medical	15	1	2	2	1	1	1	1	3
Orthopedic	9	3	3	0	0	0	2	0	3
Neurosurgery	5	2	1	0	0	0	1	0	2
Paediatric	5	1	2	0	0	0	0	0	1
ICU	5	0	0	0	0	0	0	0	1
Gynaecology	2	1	0	0	0	0	0	0	2
ENT	1	0	0	0	0	0	0	0	0
Total	**65**	**20**	**14**	**2**	**1**	**3**	**5**	**3**	**25**

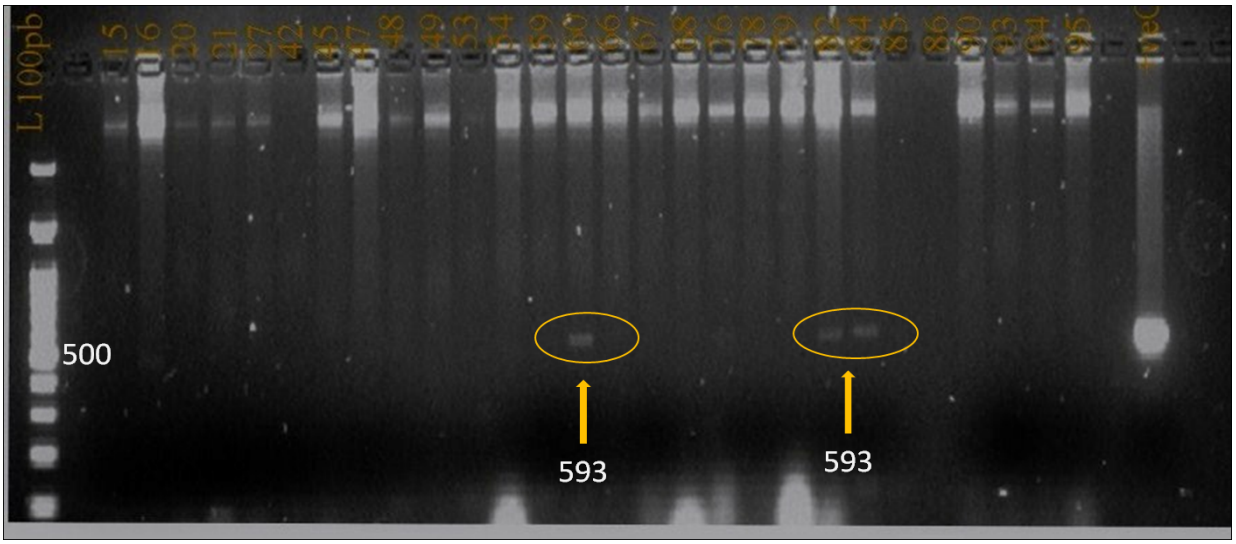
Table 3.4d: Comparison of the antibiotic resistance rates of *MBL* and *ESBL* gene PCR-positive and PCR-negative among *P. aeruginosa* isolates (n= 65)

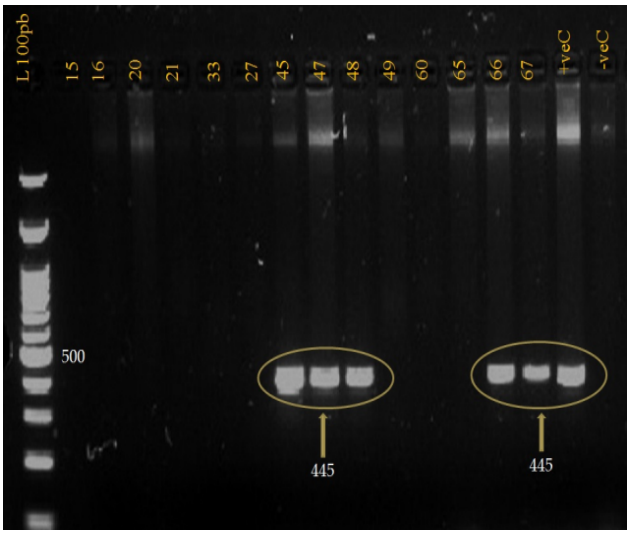
Antibiotics		Number (%) of resistant isolates				
		Total isolates (n = 65)	Non MBL producer (n =24)	MBL producer (n = 41)	Non ESBL producer (n = 32)	ESBL producer (n = 33)
β-lactams	Imipenem	65(100%)	24(100%)	41(100%)	32(100%)	33(100%)
	Meropenem	65(100%)	24(100%)	24(100%)	32(100%)	33(100%)
	Ceftazidime	62(95%)	22(92%)	41(100%)	29(91%)	33(100%)
	Piperacillin/ Tazobactam	39(60%)	8(33%)	31(76%)	6(19%)	33(100%)
	Aztreonam	41(63%)	11(46 %)	30(73%)	8 (25%)	33(100%)
Non- β-lactams	Amikacin	41(63%)	20(83%)	21(5%)	20(63%)	21(64%)
	Gentamicin	61(94%)	21(88%)	40(98%)	28(88%)	33(100%)
	Ciprofloxacin	60(92%)	20(83%)	40(98%)	27(84%)	33(100%)
	Colistin	2(3.0%)	2(8.0%)	0(0%)	2(6.0%)	0(0%)

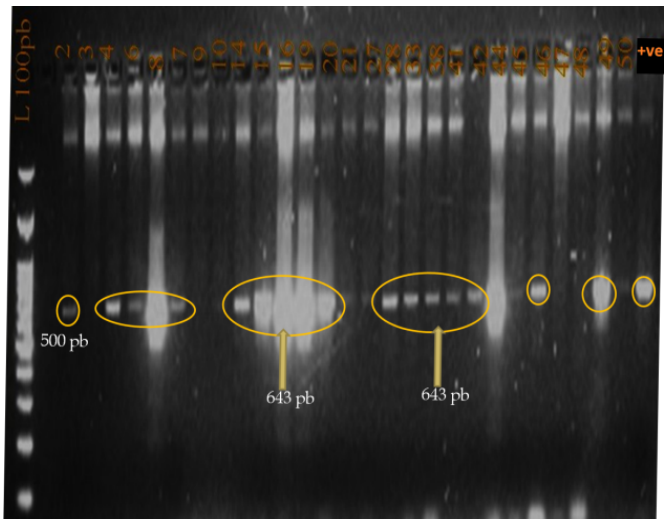
Figure 3.4 Metallo beta lactamase gene identification Gel pictures:

1. CLASS A GENES:

1.1 2.0% gel electrophoresis for *CTX-M* gene



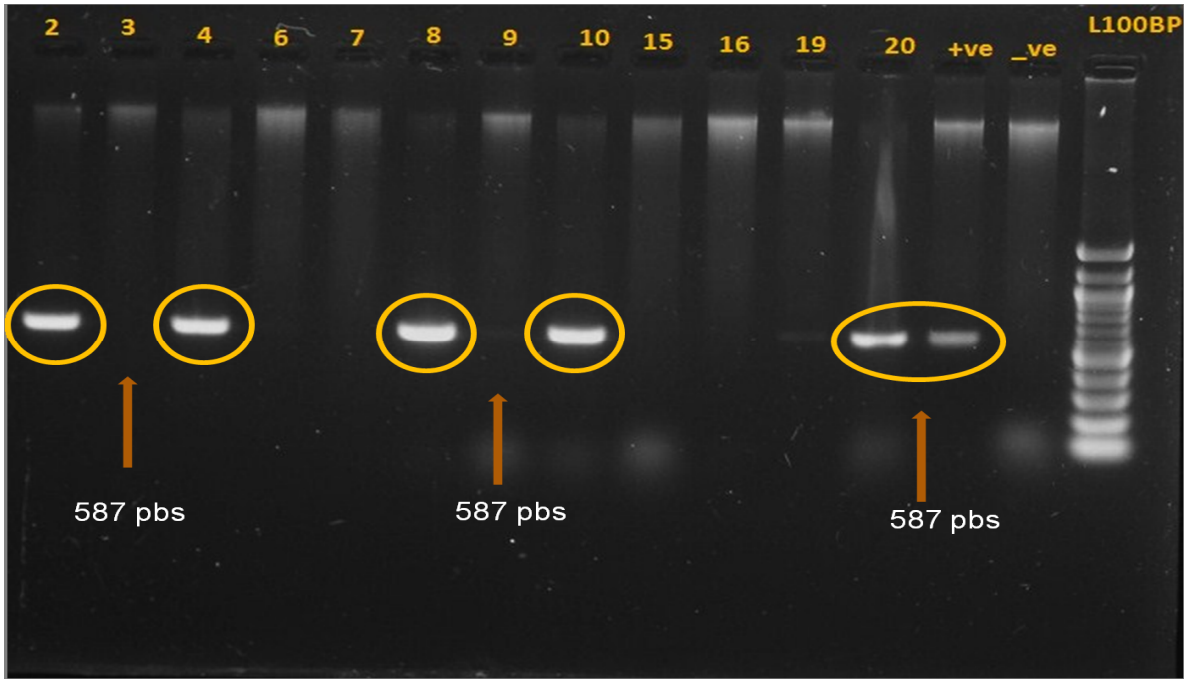




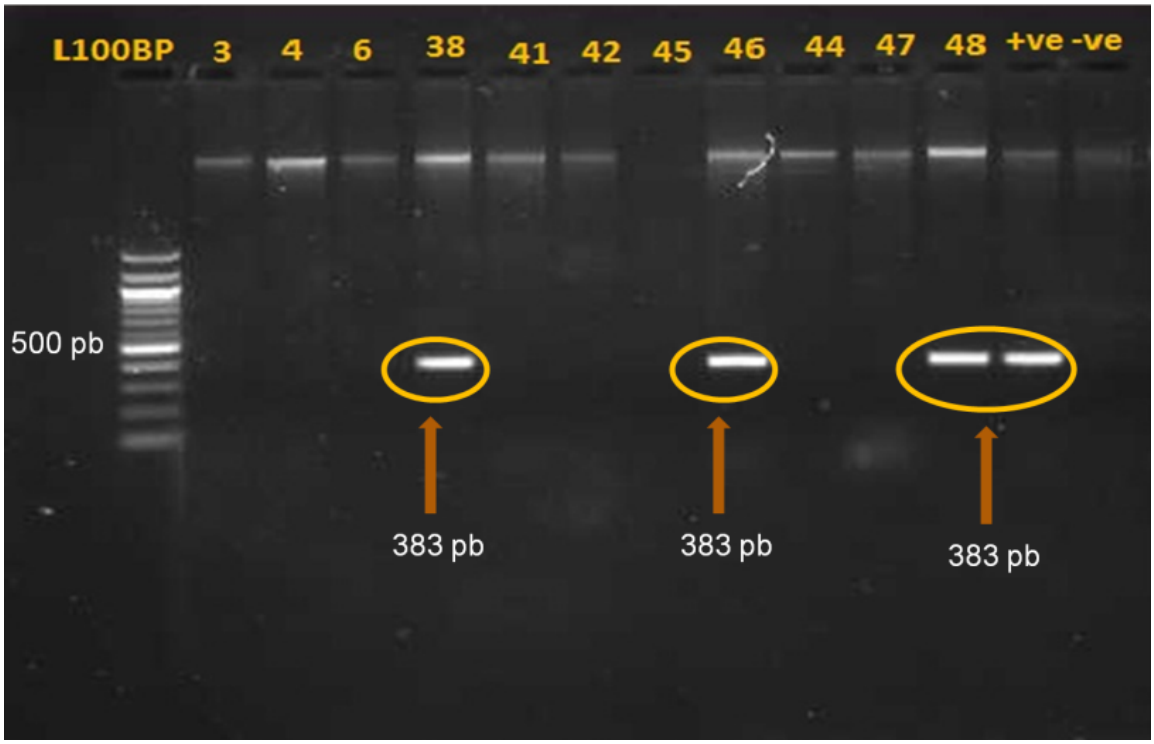
1.2 2% gel electrophoresis for *TEM* gene	1.3 2.0%gel electrophoresis for *VEB* gene

2. CLASS B GENES

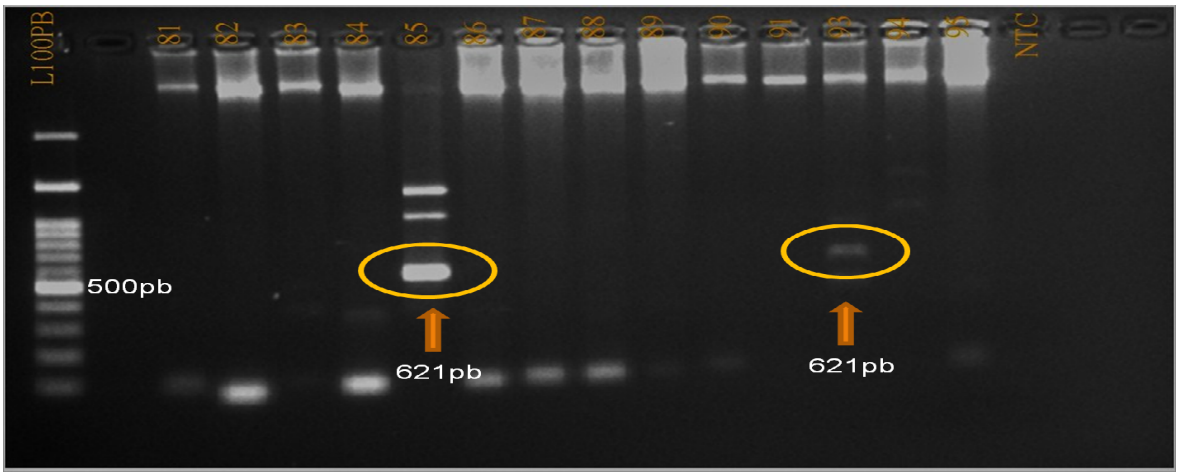
2.1 2.0% Gel electrophoresis for *IMP* gene



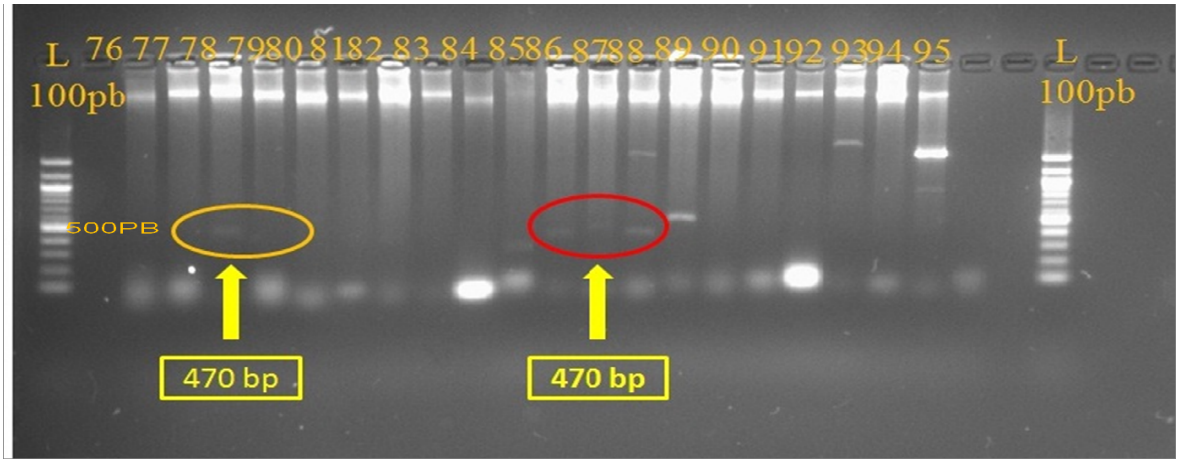
2.2 2.0% Gel electrophoresis for *VIM* gene



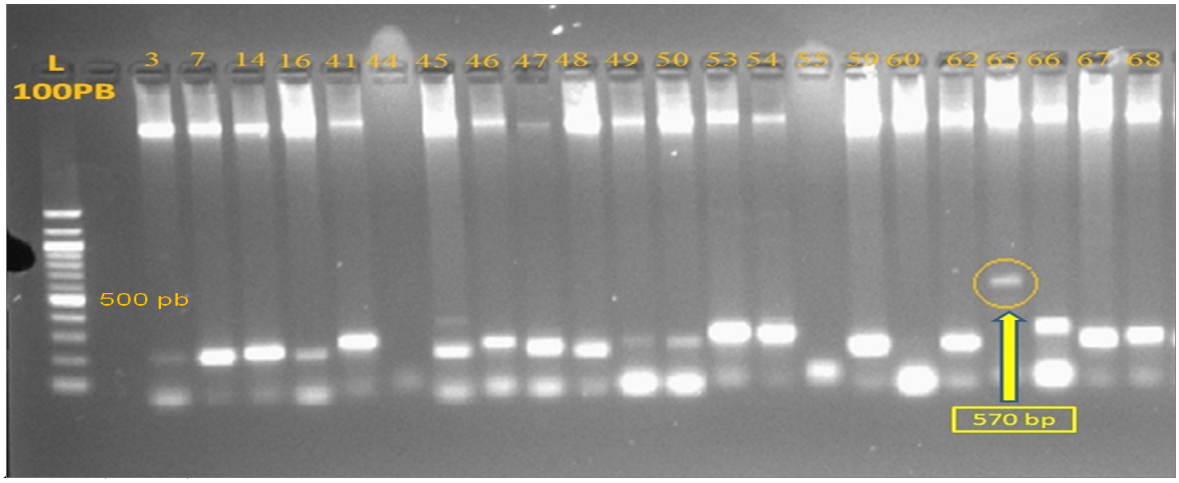
2.3 2.0% gel electrophoresis for *NDM* gene



2.4 2.0% gel electrophoresis for *GIM* gene



2.5 2.0% gel electrophoresis for *SIM* gene



Chapter 4

Discussion

4.1 Overview of antibiotics resistant profile of *P. aeruginosa* clinical isolates

P. aeruginosa is the leading cause of pneumonia and septicemia with attributable deaths reaching 30% in immunocompromised patients (Scarff & Goldberg, 2008). *P. aeruginosa* is an important pathogen associated with serious healthcare-associated infections and is an important cause of morbidity and mortality among hospital patients (Hauser & Sriram, 2005). This organism is a highly prevalent opportunistic pathogen. One of many concerning characteristics of *P. aeruginosa* is its low antibiotic susceptibility. It has a propensity to develop resistance during therapy, even evolving an MDR phenotype. *P. aeruginosa* develops resistance to antibiotics either through the mutation of chromosomally encoded genes or by the horizontal gene transfers of antibiotics resistant determinants, therefore serial culture and susceptibility testing is advisable during therapy (Li & Nikaido, 2009). In the last few decades, the incidences of multidrug-resistant *P. aeruginosa* strains have been increasing worldwide thus limiting therapeutic options (Bonomo & Szabo, 2006). The incidence of multidrug resistant *P. aeruginosa* often changes dramatically between communities and hospitals in the same area, and among many patients and populations in the same hospital (Wroblewska *et al.*, 2006; Marcel *et al.*, 2008). These variations faced by physicians in clinical treatment are responsible for effective clinical management on the antibiotic choice to be effectively and correctly used according to the data on the prevalence and resistance pattern.

Treatment of *P. aeruginosa* infections is complicated by the intrinsic and acquired resistance of this bacterium to many commonly used antimicrobial agents (Lister*, et al.*, 2009). Therapeutics of *P. aeruginosa* is a challenge for treatment of healthcare-associated infections and the choice of suitable antipseudomonal agents for treatment is significant in optimizing clinical effect (Micek *et al.*, 2005; Lister *et al.*, 2009). Surveillance programs should be conducted periodically to evaluate the

susceptibility pattern of these bacteria against prescribed antibiotics since the emergence of resistance remains silent for periods and takes time to become apparent. These antibiotics should be prescribed with caution so that antibiotic resistance can be avoided (Jombo *et al.*, 2010). The *in vitro* sensitivity studies also help in the cost-effective prescription of different brands of the antibiotic to different patients with varying socioeconomic backgrounds (Masood, 2010). Overall, susceptibility patterns in different regions should be conducted so that the profiles of specific regions are maintained and monitored accordingly. This will therefore act as a guide toward the formulation of a comprehensive monitoring program of chemotherapy. For this reason, testing of the antimicrobial susceptibility pattern is required not only for therapy but also to monitor the spread of resistant organisms or resistance genes throughout the hospital and community. In some cases, the presence of a resistance gene is highly predictive for clinical outcome of antimicrobial therapy. Therefore, determination of the antimicrobial susceptibility pattern and identification of the causes of this drug resistance in our Medical Centre are crucial to define the molecular and genetic bases of the MDR mechanisms in order to combat these infectious diseases.

In this study, 88 *P. aeruginosa* clinical isolates were collected from the University of Malaya Medical Centre. Most of the isolates were collected from urine specimens, which accounted for 47 of the total isolates. This is not surprising as almost all patients going in for major surgery are catheterized. This was supported by Olayinka *et al*. (2004) who reported that the use of indwelling catheters leads to an inherent risk for infection (Olayinka *et al.*, 2004). In this study, only 19 wound specimens were collected because there are many complications after operative surgery, which use broad-spectrum antibiotics as prophylaxis. These are mostly colonized by *P. aeruginosa* in the lower intestinal tract. Colonization of this

bacterium also depended on aseptic dressing and good clinical management (Humphreys, 2009; Zuanazzi *et al.* ,2010). Twelve specimens were collected from indwelling medical devices that represented healthcare-associated infections for patients admitted in intensive care units during the period of the medication in the hospital as healthcare-associated infections as shown in Tables 3.1a and 3.1b. Healthcare-associated infections caused by *P. aeruginosa* pose a serious problem in clinical settings due to the high prevalence of infection, particularly in intensive care units and cases of multi-drug resistance (Falagas *et al.*, 2005a; Lagatolla *et al.*, 2006).

A number of studies have been performed to determine the effectiveness of several antibiotics to these bacteria (Livermore *et al.*, 2001; Nadeem Sajjad Raja, 2007; Olayinka *et al.*, 2004; Wroblewska *et al.*, 2006). In the current study, piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, meropenem, imipenem and colistin were chosen to represent the primary antibiotic classes used for treatment of *P. aeruginosa* infections. The susceptibility pattern of the above mentioned antipseudomonal drugs against *P. aeruginosa* clinical isolates were determined using E-test® as a quantitative measure for antimicrobial susceptibility. The antibiotic resistance analysis showed increased resistance to gentamicin (94.0%), followed by ciprofloxacin and ceftazidime (92%), imipenem (74.0%), amikacin (66.0%), piperacillin/tazobactam (58.0%), aztreonam (56.0%) and colistin (7.0%) compared to *P. aeruginosa* ATCC 27853 as is illustrated in Figure 3.2e. These findings indicated that *P. aeruginosa* isolates exhibited high resistance to most antimicrobial agents examined, except colistin, which showed low levels of resistance and was still active against this organism. These results are in agreement with many studies that demonstrated high rates of antibacterial resistance towards *P. aeruginosa,* where they showed similar

resistance to the antibiotics examined in this study (El Amin *et al.*, 2005; Marra *et al.*, 2006; Sivanmaliappan & Sevanan, 2011). Raja & Singh (2007) and Pathmanathan *et al.* (2009) showed a low rate of resistance in the last three years (Raja & Singh, 2007; Pathmanathan *et al.*, 2009). Whereas, the current study disagrees with previous studies except in the case of colistin. Inappropriate and incorrect administration of antimicrobial agents in empiric therapies could be one of the reasons for this alarming phenomenon. This confirmed the increasing rate of resistance due to the empirical therapy of *P. aeruginosa* infection and high pressure of this drug choice. Colistin has become the drug choice for MDR Pseudomonal infections, in spite of its very limited pharmacokinetic and pharmacodyanamic data being available (Tam *et al.*, 2005; Sobieszczyk *et al.*, 2004).

4.2 Resistant mechanism of *P. aeruginosa* clinical isolates and gene expression trends

In the present study, we aimed to evaluate the chromosomally encoded resistance mechanisms among 88 *P. aeruginosa* clinical isolates by observing the expression levels of *ampC* cephalosporinase, the multidrug efflux pumps, the *oprD* outer membrane porin and the penicillin binding protein (*PBP2*, *PBP3*) that contribute to both intrinsic and acquired resistance and to study the relationship with antibiotics resistance patterns. Nucleic acid-based detection systems present rapid and sensitive methods to detect the presence of resistance genes and play a critical role in the elucidation of resistance mechanisms.

4.2.1 Expression of multidrug efflux pump genes

The genome analysis of *P. aeruginosa* revealed the presence of 11 RND-type efflux pumps (www.pseudomonas.com). The MexAB–OprM, MexCD–OprJ, MexEF–OprN and MexXY pumps have been shown to be of clinical importance. These contribute to the development of multiple resistances to all strategic antipseudomonal antibiotics and are mediated genetically by different components of efflux systems. It also belongs to the resistance–nodulation–division (RND) family. They were investigated in *P. aeruginosa* to determine the respective mRNA transcription levels in *P. aeruginosa* clinical isolates. These were measured by quantitative real time PCR on CFX96™ PCR detection systems (BioRad, Hercules, CA) targeting the *mexB*, *mexY*, *mexZ, mexCD* and *mexEF* genes.

In the present study, *mexB* mRNA expression of 88 *P. aeruginosa* clinical isolates as a marker for *MexAB-OprM* expression showed overexpression in up to 78% in the isolates that were resistant to piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, meropenem and imipenem drugs as shown in Table 3.3e. This number is much higher than those obtained previously by multicenter Spanish study for isolates resistant to all first line agents (all β-lactams and aminoglycosides) that showed overexpression of *mexB in* only 25% of isolates (Cabot *et al.*, 2011). Another study conducted at a tertiary teaching hospital located in São Paulo, Brazil showed overexpression of *mexB* gene in 27.1% of *P. aeruginosa* isolates that were resistant to antimicrobials and demonstrated overexpression of *mexB* in 62.5% of the isolates that were meropenem resistant (Xavier *et al.*, 2010). This study confirmed the contributions of MexAB–OprM overexpression to intrinsic resistance to a number of antimicrobials including piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, and carbapenems antibiotics.

MexXY-OprM is the second efflux pump system that contributes to intrinsic and acquired resistance to antimicrobials, and is capable of pumping out amikacin, ciprofloxacin, gentamicin, cefotaxime, meropenem, cefepime and erythromycin (Tomas *et al.*, 2010). In the current study, mRNA expression analysis in 88 *P. aeruginosa* clinical isolates demonstrated that more than 80% showed up-regulation of *mexXY-oprM* among the isolates that were resistant to gentamicin, amikacin, ciprofloxacin, ceftazidime, piperacillin/tazobactam, meropenem and imipenem as shown in Table 3.3e. These findings are in agreement with a previous study carried out by Hocquet *et al.* (2006) who found overexpression of *mexXY-oprM* in 84% of the isolates that were resistant to aminoglycosides, fluoroquinolones, ceftazidime, piperacillin/tazobactam and carbapenems (Hocquet *et al.*, 2006). This was also shown in many other studies where reduced susceptibility to various antibiotics known to be substrates for the MexXY-OprM pump were demonstrated (Wolter *et al.*, 2004; Islam *et al.*, 2004; Hocquet *et al.*, 2006; Llanes *et al.*, 2004; Hocquet *et al.*, 2008; Xavier *et al.*, 2010; Cabot *et al.*, 2011).

In the results of this study, the mRNA expression of *P. aeruginosa* clinical isolates showed 90%, 86% and 84% overexpression of *mexY* gene in the isolates that were resistant to amikacin, gentamicin and ciprofloxacin respectively. Of those, more than 96% also showed reduced *oprD* transcription (Table 3.3e). These results are in agreement with Xavier *et al*. (2010), who reported that 86.7% of the isolates that were resistant to amikacin, gentamicin and ciprofloxacin expressed *mexY* and of those, 79.2% also showed reduced *oprD* transcription (Xavier *et al.*, 2010). In addition, the up-regulation of MexXY-OprM was highly significant isolates that were resistance to aminoglycoside drugs, particularly amikacin and gentamicin. These findings were also in concordance with previous studies by Poole (2007), Vila and Martines (2008) and Toma *et al.* (2010). They reported that most of the

aminoglycoside drugs were significant substrates for MexXY-OprM (Poole 2007; Tomas *et al.*, 2010; Vila & Martinez, 2008). In addition, the current study was consistent with other studies, which suggested the up-regulation of mexXY also conferred reduced susceptibility to aminoglycosides detected in 6 of the 7 clinical isolates and was responsible for the decreased antimicrobial efficacy of aminoglycoside among clinical isolates. This is due to the longer aminoglycoside treatment periods in a clinical setting where there is a higher risk of occurrence of resistance and possibly therapeutic failure (Hocquet *et al.*,2008; Cabot *et al.*, 2011; Hocquet *et al.*, 2003; Wolter *et al.*, 2004). The highest prevalence of *mexY* overexpression (84%) was found among isolates that were resistance to ciprofloxacillin. These incidences of isolates hyperproducing MexXY-OprM were influenced by the use of aminoglycosides and fluoroquinolones drugs (Hocquet *et al.*, 2008; Islam *et al.*, 2004). Besides that, the *mexY* overexpression (80%) was noted in isolates that were resistant to meropenem and imipenem (Table 3.3e). These support the results of the previous studies which emphasized the capability of *P. aeruginosa* to develop dual resistance to carbapenems and fluoroquinolones due to independent mechanisms of resistance of different classes of antibacterial agents and stress the requirement for the judicious utilization of treatment when dealing with *P. aeruginosa* to avoid development of multidrug resistance (Wolter *et al.*, 2004; Islam *et al.*, 2004).

The mRNA expression analysis in *P. aeruginosa* isolates that were resistant to meropenem, imipenem, ciprofloxacin, ceftazidime and amikacin demonstrated overexpression of *mexZ* gene ranging from 61% to 69%. In addition, mRNA expression levels in isolates resistant to the above antibiotics were also associated with high overexpression of *mexY* gene ranging from 80% to 90%. Previous studies confirmed that mutation in genes encoding repressors and activators of efflux

pumps lead to pump expression with the most frequent target being the repressor gene *mexZ* leading to MexXY-OprM overproduction (>80%) which confers resistance to aminoglycosides, quinolones and carbapenem drugs (Wolter *et al.*, 2004; Islam *et al.*, 2004; Henrichfreise *et al.*, 2007).

In this study, we observed a significant down-regulation of *oprM* in the isolates that were resistant to amikacin and gentamicin (>62%) as shown in Table 3.3e. However, >86% of and 72% of the isolates overexpressed MexXY and MexAB-OprM. These findings were in consent with others studies, which confirmed that the MexXY efflux system lacks a cognate outer membrane protein and that OprM can substitute for the missing channel protein function. The MexXY and MexAB-OprM pump contribute to aminoglycoside resistance in a low ionic strength environment, which confirms the over expression of MexAB-OprM. MexAB-OprM expression is the growth phase is regulated but MexXY is not, which sustains the propositions that *OprM* has a second promoter, even though the *oprM* mRNA levels were even lower than the *P. aeruginosa* wild type strain. The MexXY and MexAB-OprM systems are possibly co-regulated as they expressed high levels of *mexY*. This is because *oprM* has a secondary promoter and MexXY utilized outer-membrane proteins other than OprM, such as OpmG, OpmI and OpmH (Evans & Poole, 1999; Evans *et al.*, 2001; Li *et al.*, 2003a; Okamoto *et al.*, 2002; Jo *et al.*, 2003).

The mRNA expression analysis in *P. aeruginosa* isolates that were resistant for ciprofloxacin demonstrated 63% down-regulation for *oprM*, which was associated with 78% overexpression of *mexB* and 84% hyperexpression of *mexY* genes as illustrated in Table 3.3e. This support that *mexY* require OprM in order to form a functional pump for export the antibiotics. OprM was proposed because it is always expressed at low but detectable levels from a secondary promoter independent of MexAB (Kohler *et al.* ,1999c; Li *et al.*, 2000b). This also concurs with Aires *et al.*

(1999) and Mine *et al*. (1999), who reported that MexXY efflux system share the OprM channel with MexAB thus confirming the significance of efflux mechanisms in the isolates resistant to ciprofloxacin (Aires *et al.*, 1999; Mine *et al.*, 1999). This is due to MexXY efflux pump lacking an outer-membrane protein of its own, and OprM can substitute the missing function and selected in fluoroquinolone resistant strains demonstrating a multidrug resistance phenotype that was originally attributed to reduced outer membrane permeability (Masuda*, et al.*, 2000a).

MexEF-OprN is the third efflux system which can export fluoroquinolones (Kohler *et al.*, 1999b; Maseda *et al.*, 2000). Fluoroquinolones are the choice for mutant selection of multidrug efflux pumps *in vivo* and *in vitro*, including MexEF-OprN (Join-Lambert *et al.*, 2001). This supports our results, where mRNA expression analysis in isolates that were resistant to ciprofloxacin showed that 68% of them overexpressed MexEF-OprN as shown in Table 3.3e. Overproduction of MexEF-OprN assists in development of resistance to the carbapenems due to regulatory links between this pump and the porin, OprD (Ko¨hler *et al.*, 1997). The results of this study confirm a dual resistant phenotype in which the overexpression of MexEF-OprN was noted in 68% of the isolates that were resistant to both imipenem and ciprofloxacin drugs and in 70% of isolates that were resistant to meropenem as shown in Table 3.3e. This dual resistance increases an anxiety concerning the promotion of carbapenem resistance during the utilization of fluoroquinolones. The present study results showed that 84% of the isolates that were resistant to ciprofloxacin drugs were overexpressed *mexY* which correlated with 68% of the same isolates showing upregulated expression of *mexEF* (Table 3.3e). These findings are consistent with others that confirm function of overproduction of *MexEF-OprN* in dual resistance of these strains to the fluoroquinolones as being attributed to up-regulation of the MexXY efflux pump (Poole & Srikumar, 2001;

Wolter *et al.*, 2004). On the other hand, the function of MexXY in the isolates that were resistant to fluoroquinolones has gathered interests, even though reports demonstrate a relationship between overproduction of this pump in laboratory-selected mutants with reduced sensitivity to the fluoroquinolones (Masuda *et al.*, 2000a; Jeannot *et al.*, 2005; Islam *et al.*, 2004).

Several studies have showed the participation of the MexEF-OprN, MexCD-OprJ, and MexAB-OprM efflux systems in fluoroquinolone-resistant clinical isolates (Schweizer, 2003; Jombo *et al.*, 2010; Li & Nikaido, 2009). These findings are consistent with the results of this study that illustrated 68%, 48% and 78% of the isolates that were resistant to ciprofloxacin overexpressed the *mexEF, mexCD* and *mexB* genes. Therefore, the dual resistance to the carbapenems and fluoroquinolones remains a big problem because carbapenems and fluoroquinolones remains the main antimicrobials for treating infections due to multidrug-resistant *P. aeruginosa*, but the development of resistance may significantly compromise their efficacy.

Previous studies reported that fluoroquinolones, β-lactams and meropenem are substrates for the MexCD-OprJ system, although this efflux pump is not constitutively expressed under usual growth circumstances (Maseda *et al.*, 2000; Poole *et al.*, 1996a). This coincides with the results of this study, in which the overexpression *mexCD-oprJ* was noted in 48% of the isolates that were resistant for ciprofloxacin and ceftazidime, in 55% of the isolates that were resistant for aztreonam and meropenem and in 54% of the isolates that were resistant to imipenem as is illustrated in Table 3.3e. These numbers are higher than previously, reported where only 31% of the carbapenem resistant isolates overexpressed the MexCD-OprJ efflux pump (Rodriguez-Martinez *et al.*, 2009). The mechanisms leading to carbapenem resistance appear to be more complex and are very likely

multifactorial, involving overproduction of AmpC or overexpression of the efflux pumps MexAB-OprM, MexXY-OprM and MexCD-OprJ (El Amin *et al.*, 2005). In the Rodriguez-Martinez *et al*. (2009) study no isolate overexpressed all three efflux pumps, whereas our results demonstrated up-regulation for AmpC in >65% of the isolates that were resistant to carbapenems and more than 71% of these isolates also showed overexpression of *mexB* and *mexY*. In addition, up to 55% of the isolates that were resistant to carbapenems demonstrated over expression of *mexCD* as shown in Table 3.3e.

OprM functions with all RND transporter/membrane fusion protein complexes studied including MexCD and MexEF, which is expressed at low levels (Gotoh *et al.*, 1998b; Gotoh *et al.*, 1998a; Maseda *et al.*, 2000). Another study demonstrated the functional replacement between OprJ and OprM, and the role of the inner membrane-associated components MexCD (Srikumar *et al.*, 1997). The same observation was made in this study where *oprM* down-regulation was associated with overexpression of *mexCD* and *mexEF* in the resistant isolates for the examined antibiotics (Table 3.3e). These findings also concur with other studies that demonstrated that OprM could function with all RND transporter/MPF complexes studies to date, including MexCD and MexEF in the efflux pumps of the antibiotics (Gotoh *et al.*, 1998a; Masuda *et al.*, 2000b).

4.2.2 Analysis of AmpC expression

AmpC type β-lactamases (also called cephalosporinases) are commonly found from extended-spectrum cephalosporin-resistant Gram-negative bacteria. *AmpC* type β-lactamases are encoded on the chromosome of many Gram-negative bacteria, notably *P. aeruginosa;* they can be hyperexpressed through derepression (Jacoby, 2009). AmpC overproduction by *P. aeruginosa* can be difficult to analyze owing to

the multiple resistance mechanisms usually expressed by MDR *P. aeruginosa,* but AmpC overproduction is major factor in persistent bacteremia and inappropriate therapy. Furthermore, AmpC overproductions play also an important role in the multi-drug resistance phenotype among *P. aeruginosa*. The results of the current study demonstrated 65% of the isolates that were resistant to antibiotics examined exhibited up-regulated *ampC* gene expression as shown in Table 3.3e. This is considered much higher than the results obtained by Cabot *et al*. (2011) study that demonstrated only 24.2% of isolated exhibited *ampC* overexpression. One factor possibly contributing to this difference is the resistance level for the different antibiotics used as our isolates showed higher rates of antimicrobial resistance. Another important issue to consider for an understanding of resistance dynamics are the interconnections between the different resistance mechanisms. Previously reported prevalences of the different combinations of resistance mechanisms are *mexY* (13.2%), *mexB* (12.6%), *mexF* (4.2%) and *mexD* (2.2%) overexpression where in our study the values were *mexY* (82%), *mexB* (73%), *mexF* (68%) and *mexD* (48%) considerably higher as shown in Figure 3.3.

The derepression of the chromosomal AmpC cephalosporinase and the up-regulation of the MexAB-OprM, *MexCD-OprJ*, MexEF-OprN and MexXY-OprM efflux systems confer fluoroquinolone resistance, and resistance to some β-lactams and aminoglycosides (Henrichfreise *et al.*, 2007). This is clear with results of the current study which demonstrated that more than 60% of the isolates that were resistant to antimicrobial agents mentioned above showed up-regulation of an *ampC* and this was associated with more than 71%, 76%, 63% and 47% of these resistant isolates overexpressing *mexB*, *mexY*, *mexEF* and *mexCD* mRNA respectively, as shown in Table 3.3e.

It has been reported that AmpC β-lactamase is able to cause very little activity alone against carbapenems but when combined with other resistance mechanisms can play a considerable role in reducing susceptibility to carbapenems (Okamoto *et al.*, 2001; Quale *et al.*, 2006). Additionally, the association of distinct mechanisms such as AmpC overproduction and porin down-regulation also play an important role in the carbapenem resistance phenotype among *P. aeruginosa* isolates (Xavier *et al.*, 2010). Previous studies demonstrated interaction of down-regulation of OprD and up-regulation of AmpC which appeared to be the cause for decreased meropenem and imipenem susceptibility, while further efflux pump overexpression leads to full meropenem resistance (Henrichfreise *et al.*, 2007; Tomas *et al.*, 2010; Cabot *et al.*, 2011). In the present study, 66% of the isolates that were resistant to carbapenems overexpressed *ampC*. Of those, 100% showed decreased *oprD* expression. In the study of Xavier *et al*. (2010), 52.2% were resistant to carbapenems and in those isolates 20% overexpressed AmpC. Of these, 90.0% showed decreased *oprD* expression and this is an important finding. This is related to interplay of different resistance mechanisms that play an important role in the carbapenem resistance phenotype among the *P. aeruginosa* clinical isolates studied as shown in Table 3.3e.

This study further confirms that chromosomal AmpC β-lactamase confers resistance to most cephalosporins and penicillins (piperacillin/tazobactam) in *P. aeruginosa* with high significance ($p < 0.05$) as shown in Table 3.3e and 3.3f. This finding is in line with other studies, which reported that *P. aeruginosa* clinical isolates that were resistance to piperacillin/tazobactam drug showed overexpression of the chromosomal AmpC β-lactamase gene (Quale *et al.*, 2006; Xavier *et al.*, 2010; Juan *et al.*, 2006). In these studies, the up-regulation of the *P. aeruginosa*

chromosomal cephalosporinase ultimately leads to constitutive hyperexpression of AmpC and high-level antipseudomonal β-lactam resistance.

4.2.3 Analysis of *OprD* expression

The mechanism of carbapenem (imipenem and meropenem) resistance is usually multifactorial, and may be mediated via several distinct mechanisms involving overexpression of efflux pump, AmpC overproduction or decreased levels of the outer membrane porin OprD (Pfeifer *et al.*, 2010; Wang *et al.*, 2010; Ozer *et al.*, 2012). The loss of OprD has been reported as the most consistent finding in carbapenem-resistant isolates (Pai *et al.*, 2001). As reported by Quale *et al*. (2006), down-regulation of *oprD* was found in all isolates resistant to imipenem and meropenem, because the entry of carbapenems involves the outer membrane protein OprD, and its decreased expression is commonly shown in carbapenem-resistant isolates (Quale *et al.*, 2006). There are several reports of antibiotic resistance acquired through loss or functional change of porins in a large number of organisms, such as *Neisseria gonorrhoeae*, *E. coli*, *P. aeruginosa*; *Enterobacter aerogenes* and *Klebsiella pneumoniae* (Poole 2002; Achouak *et al.*, 2001). Farra *et al*. (2008) reported that decreased transcription of *oprD* was observed equally in transconjugants and in clinical strains that were imipenem resistant and possibly observed as elevated MICs noted for meropenem (Farra *et al.*, 2008). In the current study, 100% of the isolates that were resistant to imipenem and meropenem showed down-regulation of *oprD* (Table 3.3e). The results reported in the this study are also consistent with previous investigators (Giske *et al.*, 2008a; Gutierrez *et al.*, 2007; Livermore, 2001a; Cabot *et al.*, 2011), who reported that the mechanism of resistance to imipenem and meropenem in clinical strains is associated with low expression of *oprD* which increases the MIC level to these drugs.

4.2.4 Penicillin-binding proteins (PBPs)

In *Acinetobacter baumannii*, the reports on resistance mechanisms mainly focused on outer membrane impermeability, production of beta lactamases and production of efflux pumps. However, crucial to bacterial survival are penicillin-binding proteins (PBPs), which play an important function in the synthesis of peptidoglycan, a fundamental component of the bacterial cell wall. PBPs catalyze the last step of polymerization (transglycosylation) and by cross-linking the transpeptidation of peptidoglycan, the bacteria can acquire β-lactam resistance by changing their PBPs and studies showed an association between down-regulation of *PBPs* and resistance in *A. baumannii* (Vashist *et al.*, 2011). A study by El Amin *et al.* (2005) on *P. aeruginosa* clinical isolates that were resistant to imipenem and meropenem depicted that down-regulation of *oprD* did not always associate with the degree of meropenem and imipenem resistance and the divergences might not be clarified by the pattern of efflux pump gene expression or OprD alterations. For this reason, it was believed that additional mechanisms were involved in meropenem and imipenem resistance (El Amin *et al.*, 2005). Another probable resistance mechanism may be the alterations in the penicillin-binding proteins (PBPs) and PBPs are one of the most likely mechanisms for carbapenem resistance offer than enzymes (Farra *et al.*, 2008). The results of the current study demonstrated that 80% of the *P. aeruginosa* clinical isolates that were resistant to imipenem and meropenem showed down-regulation of *PBP2*, while up to 91% of these isolates showed down-regulation of *PBP3* (Table 3.3e). However, Farra *et al*. (2008) reported, in their study, that decreased expression of *PBP2* and *PBP3* was noted in all strains in comparison to *PA018SR*, except for the clinical strain, which showed increased expression of both *PBPs*. Therefore, the increased expression of *PBP2* and *PBP3* genes is possibly not significant for resistance to carbapenems

(Farra *et al.*, 2008). Previous studies reported that *PBP2* contributes significantly to β-lactam antibiotic resistance, and down-regulation of *PBP3* has effects on a number of genes in *Pseudomonas*, involved in antibiotic resistance (Blazquez *et al.*, 2006; Legaree *et al.*, 2007). The present study shows that imipenem and meropenem resistance among clinical *P. aeruginosa* isolates not only occurred due to OprD down-regulation but was also linked with down-regulation of other genes such as *PBP2* and *PBP3*. This implies that down-regulation of the *oprD* gene significantly contributes to imipenem and meropenem resistance, which increases the MIC to these drugs. While, *PBPs* can be one of the most likely mechanisms for carbapenem resistance among clinical *P. aeruginosa* isolates, the mechanisms of resistance to carbapenem (imipenem and meropenem) as a result of the interplay between diminished production of OprD, increased activity of AmpC, and several efflux systems has been illustrated to contribute high-level resistance to these agents (Quale *et al.*, 2006; Henrichfreise *et al.*, 2007).

4.3 Detection the prevalence of metallo-beta lactamase producing *P. aeruginosa*

In the current study, we aimed to evaluate the different mechanisms associated with reduced susceptibility to imipenem and meropenem compared to other examined antibiotics. The mechanism of carbapenem (imipenem and meropenem) resistance is usually multifactorial, involving overexpression of efflux, AmpC overproduction or decreased outer membrane *OprD* levels or by acquired β-lactamases, included extended spectrum types (ESBLs) and carbapenemases (Pfeifer *et al.*, 2010; Wang *et al.*, 2010; Ozer *et al.*, 2012). Therefore, this study was carried out to detect the prevalence of MBL producing *P. aeruginosa* in the University Malaya Medical Center (UMMC) in Kuala Lumpur, Malaysia, to discover the associated risk factors and probable treatment alternatives. The early detection of MBL producing *P.*

aeruginosa may avoid the future spread of these multi-drug resistant strains. In the present study, 41 out of 65 imipenem and meropenem resistant *P. aeruginosa* isolates from various clinical specimens were MBL producers. Out of 41 strains, MBL genes were amplified in 20 isolates for bla_{IMP}, in 14 isolates for bla_{VIM}, in 4 isolates for bla_{GIM}, in 2 isolates for bla_{NDM} and only in one isolate for bla_{SIM} (Table 3.4a). No bla_{SPM} genes were detected in this study. The detection of bla_{IMP} and bla_{VIM} is in agreement with a previous study that carried out at the University Malaya Medical Center (UMMC) in Kuala Lumpur, Malaysia (Khosravi *et al.*, 2010). It demonstrated that 32 out of 90 imipenem resistant *P. aeruginosa* clinical isolates tested positive for the presence of MBL genes including bla_{IMP} and bla_{VIM} and none of the isolates tested positive for other MBL genes (Khosravi *et al.*, 2010). However, another study also carried out in same medical centre showed that only one *P. aeruginosa* out of 50 imipenem resistant *P. aeruginosa* isolates was MBL gene PCR positive (Ho *et al.*, 2002). The results of this study are interesting in that this is the first study to show that imipenem and meropenem resistant *P. aeruginosa* isolates harbor bla_{SIM}, bla_{GIM} and bla_{NDM}.

P. aeruginosa isolates producing metallo-β-lactamases (MBLs) were first reported from Japan in 1991 and since then have been described in various parts of the world, including Asia, Europe, Australia, South America and North America (Poirel *et al.*, 2007).

A number of studies have been carried out regarding the prevalence of MBL-producing isolates among carbapenem resistant *P. aeruginosa* and varying percentages have been reported worldwide, ranging from 10% in Turkey (Ozgumus *et al.*, 2007) and up to 36% in Southeast Asia (Huang *et al.*, 2007). In addition, there are frequent reports from Asian and the Pacific countries, such as Malaysia, Singapore, Bangladesh, Hong Kong, Taiwan and Japan (Khosravi *et al.*, 2010;

Nasrin *et al.*, 2011; Koh *et al.*, 2004; Hemalatha *et al.*, 2005). Previous studies pointed out that the most common and widespread acquired MBLs are those of the *IMP* and *VIM* types, which exhibit a worldwide distribution and are widespread in Asian countries (Yan *et al.*, 2001; Yatsuyanagi *et al.*, 2004; Khosravi *et al.*, 2010; Hwa *et al.*, 2009). This is in agreement with the results of this study that found most imipenem and meropenem resistant isolates harbor *IMP* and *VIM* types.

Recently, the New Delhi metallo-β-lactamase (NDM) has been reported from diverse locations such as the UK, the USA, Japan, India, Singapore, Australia and most recently the Middle East and this represents an emerging public health threat (Muir & Weinbren, 2010; Karthikeyan *et al.*, 2010; Rolain *et al.*, 2010). NDM is contributing resistance to almost all β-lactam antibiotics, including carbapenems, and was recently identified in *Escherichia coli* and *Klebsiella pneumonia* isolates from a Swedish patient who visited New Delhi, India (Yong *et al.*, 2009). Kumarasamy *et al*. (2010) have lately reported the appearance and spread of 180 cases of patients infected with bacteria carrying the NDM encoding gene from India, Pakistan and the UK. Fascinatingly, most patients from the UK had journeyed to Pakistan or India within 1 year and had been hospitalized in these countries, indicating that these organisms were acquired from a local source in Asia (Kumarasamy *et al.*, 2010).

In 2010, other cases were reported worldwide, including in the USA, Canada, Europe, Japan, Africa, Oman and Australia (Rolain *et al.*, 2010). Consequently, the detection of two NDM positive isolates in this study suggested possibilities of spread via international tourists in this country. All 41 MBL producing *P. aeruginosa* strains were resistant not only to carbapenems (imipenem and or meropenem), but also to ceftazidime, which was used as a marker for MBL production. Furthermore, the MBL producing isolates were found to be most

resistant to piperacillin-tazobactum (76%), followed by aztreonam (73%) and it was found that the MBL producer *P. aeruginosa* strains were more resistant to other antimicrobial agents than non-MBL producers (Table 3.4d). These results are in concurrence with those by Navneeth *et al*. who reported that 100% of isolates were resistance to the piperacillin/tazobactum combination and demonstrated that MBLs confer resistance not only to carbapenems but also to other β-lactams and are poorly inhibited by the presence of β-lactamase inhibitors such as tazobactam, clavulanic acid and sulbactum (Navaneeth *et al.*, 2002). The results in this study also coincide with Hirakata *et al*. (1998), who reported potent antimicrobial activity of aztreonam against MBL positive isolates (Hirakata *et al.*, 1998). This confirms that *P. aeruginosa* strains harboring MBL genes have emerged in our health facility, which needs more attention in infection control measures.

The present study demonstrated that 33 out of 65 of the imipenem and meropenem resistant *P. aeruginosa* isolates were positive for ESBL genes. Out of these 33 isolates, ESBL genes were amplified in 25 isolates for bla_{VEB}, in 5 isolates for bla_{TEM} and in 3 isolates for $bla_{CTX\text{-}M}$. No bla_{SHV} and bla_{PER} genes were detected (Table 3.4a). The presence of bla_{VEB}, bla_{TEM} and $bla_{CTX\text{-}M}$ genes in *P. aeruginosa* isolates in this study was demonstrated in Malaysia for the first time. The most frequently encountered ESBLs gene in our study was bla_{VEB} (25 of 65), which is similar to (30 of 75) ESBLs producing isolates of *P. aeruginosa* which was reported by Jiang *et al*. (Jiang *et al.*, 2006).

The first report of the presence of $bla_{CTX\text{-}M}$ ESBLs in *P. aeruginosa* was provided by Al Naiemi *et al*. (Al Naiemi *et al.*, 2006). The $bla_{CTX\text{-}M}$ ESBLs supply *P. aeruginosa* with an extra potent resistance mechanism with potential serious clinical implications, further compromising therapeutic choices. The incidence of

bla$_{CTX-M}$ has gained endemic proportion in several countries (Livermore *et al.*, 2007).

Our study reported 50% ESBL production among *P. aeruginosa* isolates examined. These findings are much higher than the results of previous studies that showed low rates, 3.7% in Woodford *et al.* (2008) study, 4.2% in Lim *et al.* (2009) study, 7.7% in Jacoby (1997) study, 20.3% in Aggarwal *et al.* (2008) study, 35.85% in Ullah *et al.* (2009), 39.41% in Mirsalehian *et al.* (2010) study, of ESBL production in *P. aeruginosa* isolates examined. These observations suggested that ESBLs are increasingly found in *P. aeruginosa*, which could also be a reservoir for the spread of these enzymes (Livermore, 1995).

ESBLs confer resistance of *P. aeruginosa* to the third generation cephalosporins in 100% of isolates examined in this study, which was much higher than that reported by Uma *et al*. In the present study, we observed an increased resistance of this ESBL producer *P. aeruginosa* isolates to the examined antibiotics except colistin, which were in accordance with previous reports (Aggarwal *et al.*, 2008; Jayakumar & Appalaraju, 2007; Arya *et al.*, 2005).

P. aeruginosa carries a notably higher mortality rate than other pneumonia pathogens. Because of its multiple mechanisms of antibiotic resistance, therapy has always been challenging. This problem has been magnified in recent years with the emergence of multidrug-resistant (MDR) pathogens often unharmed by almost all classes of antimicrobials. The increasing use of the antimicrobial and environmental stresses leading to chronic infections of individual airways is due to change in colony morphology that arises during biofilm production (von Gotz *et al.*, 2004; Häußler *et al.*, 2003; Drenkard & Ausubel, 2002). According to von Gotz *et al*., Boles *et al*., various types of colonial morphology in *P. aeruginosa* displays phenotypic modifications in motility, antibiotic resistance, biofilm formation,

adherence properties, and virulence gene expression. The creation of various phenotypic properties may possibly contribute to the persistence and pathogenic abilities of *P. aeruginosa* (von Gotz *et al.*, 2004; Boles *et al.*, 2004).

4.4 Clinical implication

Overall, *P. aeruginosa* clinical isolates in our society showed multiple resistance phenotypes leading to limited therapeutic choices. Therefore, infections caused by MDR *P. aeruginosa* have become a serious problem, especially in the healthcare-associated settings. This problem indicates the importance of performing antibiotic susceptibility testing before treatment.

Furthermore, infection with MDR *P. aeruginosa* is associated with risk factors including severity of illness, invasive devices, a bedridden state, and in hospitalized patients, lead to increased length of stay and increased therapeutic costs as well as significant morbidity and mortality. The continuous efforts of clinicians, microbiologists, pharmacists and communities should promote a greater understanding of this problem, with better hygiene and postoperative care and management.

Most of the *P. aeruginosa* clinical isolates had a high level of resistance to examined antibiotics except colistin. Therefore, the only drug of choice in MDR isolates is the colistin despite its toxicity. In addition, the search for anti-pseudomonal agents with alternative mechanisms of action is very important in this situation. In addition, it is important to practice good antibiotic prescription policies and infection control practices to prevent the spread of genes responsible for these resistances, which could result in the return to the pre-antibiotic era.

Efflux pumps contribute to both intrinsic and acquired resistance that can survive the presence of antibiotics, which may select mutants that overexpress these genes.

It was obvious that several efflux pumps genes were expressed in our isolates so these may lead to broad-spectrum resistance.

Loss of OprD reduced the susceptibility of *P. aeruginosa* to antipseudomonal carbapenems, associated with other molecular mechanisms, highlighting the complexity by which *P. aeruginosa* is able to regulate expression of resistant mechanisms, and it is difficult to relate phenotypic expression to any single mechanism. Additionally, these strains highlight the ability of *P. aeruginosa* to develop dual resistance to different classes of antimicrobial agents through independent mechanisms of resistance and highlight the need for the judicious use of therapy when dealing with *P. aeruginosa* to prevent multidrug resistance from developing.

The prevalence of ESBLs differs with geographic location and time. ESBL producing strains are generally found in areas of hospitals where antibiotic use is common and the patient's condition is critical. These resistant organisms are clinically significant as they result in increased morbidity and mortality. In addition, ESBL producing bacteria are commonly resistant to several classes of antibiotics, complicating therapy. Presently, carbapenems are considered the drug of choice for treatment of infections caused by ESBL-producing organisms. The undesirable use of carbapenems has been linked with the appearance of carbapenem-resistant *P. aeruginosa.* The increased prevalence of ESBLs is almost certainly due to the increased use of expanded spectrum cephalosporins in hospital settings. ESBLs organism producers are distinctive challenges to clinicians, scientists, clinical microbiologists and infection control specialists occupied in the discovery of new antimicrobial agents. Suitable infection control practices and barriers are essential to prevent distribution and occurrences of ESBL-producing bacteria. The infections detected in hospitalized patients caused by ESBLs illustrate

either infection acquired during hospitalization or severe community acquired infections involving hospital admission. Community-acquired infections that contribute to ESBL-producing organisms in hospitalized patients appears to be growing (Valverde *et al.*, 2008). Additionally, the long-term care facilities of residents caused by infection or colonization by the ESBL-producing organisms may provide a means for the distribution of ESBLs among hospitals and communities. In this respect, the occurrence of colonization by the ESBL-producing organisms where inhabitants stay for long periods for medication appears to be rising significantly, even though relevant data are inadequate (Nicolas-Chanoine & Jarlier, 2008).

The risk factors for the hospitalized patients towards infection or colonization by the ESBL-producing organisms are comparable to those acquired by healthcare-associated bacterial infections (Rodríguez-Baño *et al.*, 2006). A long stay in a hospital or intensive care unit (ICU), inclusion of different kinds of indwelling catheters, greater severity of clinical status, performance of surgical interventions or invasive procedures, and mechanical ventilatory support or renal replacement therapy, have all been linked to the isolation of ESBL-producing organisms from hospitalized patients (Jacoby & Munoz-Price, 2005; Pfaller & Segreti, 2006). The use of antibiotics in particular, such as fluoroquinolones or oxyimino-β-lactams, represents an important additional risk factor (Jacoby & Munoz-Price, 2005). The detection of patients colonized with the ESBL-producing organisms can be done with an examination of the cultures from the specimens gastrointestinal tract, especially by collecting swabs from the rectal area (Paterson & Bonomo, 2005). A considerably high percentage of patients who develop healthcare-associated ESBL-acquired infections associated with the preceding colonization in the gastrointestinal tract have been noticed. On the other hand, this is technically

demanding and requires the use of selective culture media, which is necessary for identification of ESBL producers among commensal *Enterobacteriaceae*. The effectiveness of infection control relating to the strategy of selective decontamination of the gastrointestinal tract of patients that are found to be colonized with ESBL-producing organisms remains a contentious subject (Paterson & Bonomo, 2005).

4.5 Conclusion

Eighty-eight clinical isolates from the University of Malaya Medical Center, Kuala Lumpur, Malaysia were identified as *P. aeruginosa* strains based on morphology, gram staining, pyocyanin production and biochemical tests by API20NE according to the standard laboratory procedures. Antibiotic susceptibility testing was done using E-test®. The antimicrobial agents tested in this work were piperacillin/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem and colistin. Most of the *P. aeruginosa* clinical isolates had a high level of resistance to all examined antibiotics except colistin. The resistance mechanisms in 88 clinical *P. aeruginosa* isolates were evaluated using real time PCR. The gene expression analysis of 88 *P. aeruginosa* isolates showed overexpression of efflux pump genes, AmpC overexpression, down-regulation of outer membrane porin OprD and penicillin binding proteins compared to *P. aeruginosa* ATCC 27853. Therefore, the multidrug resistance phenotypes in these clinical isolates were caused by the interaction of several different resistance mechanisms occurring within the same strain such as overexpression of efflux pumps, AmpC overproduction, decreased outer membrane porin OprD, alteration of penicillin binding proteins. Sixty-five of the clinical *P. aeruginosa* isolates that were resistant to imipenem and meropenem were then evaluated for detection of

MBL and ESBL genes. The results obtained with these isolates demonstrated high efflux pump overproduction, MBL and ESBL production and confirm that resistance genes have the ability to increase resistance levels to imipenem and meropenem in *P. aeruginosa.*

4.6 Future work

Multilocus Sequence Typing (MLST) could be a useful method for studying the epidemiology of MBL-producing isolates. It is also important to develop novel antimicrobial peptides and assess their possibility for use as anti effective agents and to progress it through pre-clinical studies in animal models.

Furthermore, clinical studies are required to identify the epidemiological and risk factors for MDR *P. aeruginosa* development and to determine the economic impact of these infections, as well as to design the most efficacious antimicrobial treatments and duration of therapy to maximize positive outcomes of multi drug resistant *P. aeruginosa* infections. This calls for strict precautionary measures, which includes stringent infection control practices and the judicious use of antibiotics. These important measures might overcome the challenge of high mortality posed by multi drug resistant *P. aeruginosa* and other non-fermenting bacterial pathogens.

References

Aarts, M. A. W., Hancock, J. N., Heyland, D., McLeod, R. S., & Marshall, J. C. (2008). Empiric antibiotic therapy for suspected ventilator-associated pneumonia: a systematic review and meta-analysis of randomized trials. *Crit Care Med., 36*(1), 108.

Achouak, W., Heulin, T., & Pages, J. M. (2001). Multiple facets of bacterial porins. *FEMS Microbiol Lett, 199*(1), 1-7. doi: S0378-1097(01)00127-6 [pii].

Aggarwal, R., Chaudhary, U., & Bala, K. (2008). Detection of extended-spectrum beta-lactamase in *Pseudomonas aeruginosa*. *Indian J Pathol Microbiol, 51*(2), 222-224.

Agodi, A., Barchitta, M., Cipresso, R., Giaquinta, L., Romeo, M. A., & Denaro, C. (2007). *Pseudomonas aeruginosa* carriage, colonization, and infection in ICU patients. *Intensive Care Med, 33*(7), 1155-1161. doi: 10.1007/s00134-007-0671-6.

Aires, J. R., Kohler, T., Nikaido, H., & Plesiat, P. (1999). Involvement of an active efflux system in the natural resistance of *Pseudomonas aeruginosa* to aminoglycosides. *Antimicrob Agents Chemother, 43*(11), 2624-2628.

Al Naiemi, N., Duim, B., & Bart, A. (2006). A CTX-M extended-spectrum β-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia*. *J Med Microbio, 55*(11), 1607-1608.

Allen, L., Dockrell, D. H., Pattery, T., Lee, D. G., Cornelis, P., Hellewell, P. G.*, et al.* (2005). Pyocyanin production by *Pseudomonas aeruginosa* induces neutrophil apoptosis and impairs neutrophil-mediated host defenses in vivo. *J Immunol, 174*(6), 3643.

Aloush, V., Navon-Venezia, S., Seigman-Igra, Y., Cabili, S., & Carmeli, Y. (2006). Multidrug-resistant *Pseudomonas aeruginosa*: risk factors and clinical impact. *Antimicrob Agents Chemother, 50*(1), 43-48. doi: 50/1/43 [pii] 10.1128/AAC.50.1.43-48.2006.

Amari, E., Boffi, E., Chamot, E., Auckenthaler, R., Pechère, J., & Van Delden, C. (2001). Influence of previous exposure to antibiotic therapy on the susceptibility pattern of *Pseudomonas aeruginosa* bacteremic isolates. *Clin Infect Dis, 33*(11), 1859.

Ambler, R. (1980). The Structure of beta-Lactamases. *Phil. Trans. R. Soc. B*, *289*(1036), 321-331.

Arancibia, F., Bauer, T. T., Ewig, S., Mensa, J., Gonzalez, J., Niederman, M. S.*, et al.* (2002). Community-acquired pneumonia due to gram-negative bacteria and *Pseudomonas aeruginosa*: incidence, risk, and prognosis. *Arch Intern Med, 162*(16), 1849.

Arya, M., Arya, P. K., Biswas, D., & Prasad, R. (2005). Antimicrobial susceptibility pattern of bacterial isolates from post-operative wound infections. *Indian J Pathol Microbiol, 48*(2), 266-269.

Ayala, J., Quesada, A., Vadillo, S., Criado, J., & Piriz, S. (2005). Penicillin-binding proteins of *Bacteroides fragilis* and their role in the resistance to imipenem of clinical isolates. *J Med Microbiol, 54*(Pt 11), 1055-1064. doi: 54/11/1055 [pii]10.1099/jmm.0.45930-0.

Baddour, L. M., Hicks, D. V., Tayidi, M. M., Roberts, S. K., Walker, E., Smith, R. J.*, et al.* (1995). Risk factor assessment for the acquisition of fluoroquinolone-resistant isolates of *Pseudomonas aeruginosa* in a community-based hospital. *Microb Drug Resist, 1*(3), 219-222.

Barrow, G., & Feltham, R. K. A. (2004). *Cowan and Steel's manual for the identification of medical bacteria*: Cambridge Univ Pr.

Bassetti, M., Ginocchio, F., & Mikulska, M. (2011). New treatment options against gram-negative organisms. *J Crit Care, 15*(2), 215.

Beceiro, A., Dominguez, L., Ribera, A., Vila, J., Molina, F., Villanueva, R.*, et al.* (2004). Molecular characterization of the gene encoding a new AmpC beta-lactamase in a clinical strain of *acinetobacter* genomic species 3. *Antimicrob Agents Chemother, 48*(4), 1374-1378.

Bellais, S., Leotard, S., Poirel, L., Naas, T., & Nordmann, P. (1999). Molecular characterization of a carbapenem-hydrolyzing beta-lactamase from Chryseobacterium (Flavobacterium) indologenes. *FEMS Microbiol Lett, 171*(2), 127-132. doi: S0378-1097(98)00594-1 [pii].

Bellido, F., Veuthey, C., Blaser, J., Bauernfeind, A., & Pechere, J. C. (1990). Novel resistance to imipenem associated with an altered PBP-4 in a *Pseudomonas aeruginosa* clinical isolate. *J Antimicrob Chemother, 25*(1), 57-68.

Bhavnani, S. M., Hammel, J. P., Cirincione, B. B., Wikler, M. A., & Ambrose, P. G. (2005). Use of pharmacokinetic-pharmacodynamic target attainment analyses to support phase 2 and 3 dosing strategies for doripenem. *Antimicrob Agents Chemother, 49*(9), 3944-3947.

Binnerup, S. J., Højberg, O., & Sørensen, J. (1998). Gram characteristics determined on single cells and at the microcolony level of bacteria immobilized on polycarbonate membrane filters. *J Microbiol Methods, 31*(3), 185-192.

Bisbe, J., Gatell, J. M., Puig, J., Mallolas, J., Martinez, J. A., de Anta, M. T. J.*, et al.* (1988). *Pseudomonas aeruginosa* bacteremia: univariate and multivariate analyses of factors influencing the prognosis in 133 episodes. *Rev Infect Dis, 10*(3), 629-635.

Blanc, D., Petignat, C., Janin, B., Bille, J., & Francioli, P. (1998). *Frequency and molecular diversity of Pseudomonas aeruginosa upon admission and during*

hospitalization: a prospective epidemiologic study (Vol. *4*). Oxford, ROYAUME-UNI: Wiley-Blackwell.

Blazquez, J., Gomez-Gomez, J. M., Oliver, A., Juan, C., Kapur, V., & Martin, S. (2006). PBP3 inhibition elicits adaptive responses in *Pseudomonas aeruginosa*. *Mol Microbiol, 62*(1), 84-99. doi: MMI5366 [pii] 10.1111/j.1365-2958.2006.05366.x.

Boles, B. R., Thoendel, M., & Singh, P. K. (2004). Self-generated diversity produces "insurance effects" in biofilm communities. *Proceedings of the Proc Natl Acad Sci U. S. A*, *101*(47), 16630-16635. doi: 10.1073/pnas.0407460101.

Bonnet, R. (2004a). Growing group of extended-spectrum beta-lactamases: the CTX-M enzymes. *Antimicrob Agents Chemother, 48*(1), 1-14.

Bonomo, R. A., & Szabo, D. (2006). Mechanisms of multidrug resistance in *Acinetobacter species* and *Pseudomonas aeruginosa*. *Clin Infect Dis, 43 Suppl 2*, S49-56. doi: CID38713 [pii] 10.1086/504477.

Boucher, H. W., Talbot, G. H., Bradley, J. S., Edwards, J. E., Gilbert, D., Rice, L. B.*, et al.* (2009). Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. *Clin Infect Dis, 48*(1), 1-12.

Boyd, D. A., Tyler, S., Christianson, S., McGeer, A., Muller, M. P., Willey, B. M.*, et al.* (2004). Complete nucleotide sequence of a 92-kilobase plasmid harboring the CTX-M-15 extended-spectrum beta-lactamase involved in an outbreak in long-term-care facilities in Toronto, Canada. *Antimicrob Agents Chemother, 48*(10), 3758-3764. doi: 10.1128/AAC.48.10.3758-3764.2004 48/10/3758 [pii].

Bradford, P. A. (2001). Extended-spectrum β-lactamases in the 21st century: characterization, epidemiology, and detection of this important resistance threat. *Clin Microbiol Rev, 14*(4), 933-951.

Bradford, P. A., Cherubin, C. E., Idemyor, V., Rasmussen, B. A., & Bush, K. (1994). Multiply resistant Klebsiella pneumoniae strains from two Chicago hospitals: identification of the extended-spectrum TEM-12 and TEM-10 ceftazidime-hydrolyzing beta-lactamases in a single isolate. *Antimicrob Agents Chemother, 38*(4), 761-766.

Burgess, D. S., & Hastings, R. W. (2000). Activity of piperacillin/tazobactam in combination with amikacin, ciprofloxacin, and trovafloxacin against *Pseudomonas aeruginos* by time-kill. *Diagn Microbiol Infect Dis, 38*(1), 37-41.

Bush, K. (2002). The impact of beta-lactamases on the development of novel antimicrobial agents. *Curr Opin Investig Drugs, 3*(9), 1284-1290.

Bush, K., Jacoby, G. A., & Medeiros, A. A. (1995). A functional classification scheme for beta-lactamases and its correlation with molecular structure. *Antimicrob Agents Chemother, 39*(6), 1211-1233.

Bush, K., & Mobashery, S. (1998). How beta-lactamases have driven pharmaceutical drug discovery. From mechanistic knowledge to clinical circumvention. *Adv Exp Med Biol, 456*, 71-98.

Cabot, G., Ocampo-Sosa, A. A., Tubau, F., Macia, M. D., Rodriguez, C., Moya, B., *et al.* (2011). Overexpression of *AmpC* and Efflux Pumps in Pseudomonas aeruginosa Isolates from Bloodstream Infections: Prevalence and Impact on Resistance in a Spanish Multicenter Study. *Antimicrob. Agents Chemother., 55*(5), 1906-1911. doi: 10.1128/aac.01645-10.

Cabral, J. H. M., Jackson, A. P., Smith, C. V., Shikotra, N., Maxwell, A., & Liddington, R. C. (1997). Crystal structure of the breakage-reunion domain of DNA gyrase. *Nature, 388*(6645), 903-906.

Calandra, G., Ricci, F., Wang, C., & Brown, K. (1986). Cross-resistance and imipenem. *Lancet, 2*(8502), 340-341. doi: S0140-6736(86)90027-9 [pii].

Campbell, J. I., Ciofu, O., & Hoiby, N. (1997). Pseudomonas aeruginosa isolates from patients with cystic fibrosis have different beta-lactamase expression phenotypes but are homogeneous in the ampC-ampR genetic region. *Antimicrob Agents Chemother, 41*(6), 1380-1384.

Cao, B., Wang, H., Sun, H., Zhu, Y., & Chen, M. (2004a). Risk factors and clinical outcomes of nosocomial multi-drug resistant Pseudomonas aeruginosa infections. *J Hosp Infect, 57*(2), 112-118. doi: 10.1016/j.jhin.2004.03.021S0195670104001173 [pii].

Cao, L., Srikumar, R., & Poole, K. (2004b). MexAB-OprM hyperexpression in NalC-type multidrug-resistant *Pseudomonas aeruginosa*: identification and characterization of the nalC gene encoding a repressor of PA3720-PA3719. *Mol Microbiol, 53*(5), 1423-1436. doi: 10.1111/j.1365-2958.2004.04210. MMI4210 [pii].

Cardo, D., Horan, T., Andrus, M., Dembinski, M., Edwards, J., Peavy, G., *et al.* (2004). National Nosocomial Infections Surveillance (NNIS) system report, data summary from January 1992 through June 2004, issued October 2004. *Am J Infect Control, 32*(8), 470-485.

Carmeli, Y., Troillet, N., Eliopoulos, G. M., & Samore, M. H. (1999a). Emergence of antibiotic-resistant *Pseudomonas aeruginosa*: comparison of risks associated with different antipseudomonal agents. *Antimicrob Agents Chemother, 43*(6), 1379-1382.

Carmeli, Y., Troillet, N., Karchmer, A. W., & Samore, M. H. (1999b). Health and economic outcomes of antibiotic resistance in *Pseudomonas aeruginosa*. *Arch Intern Med, 159*(10), 1127.

Castanheira, M., Toleman, M. A., Jones, R. N., Schmidt, F. J., & Walsh, T. R. (2004). Molecular characterization of a β-lactamase gene, blaGIM-1, encoding a new subclass of metallo-β-lactamase. *Antimicrob Agents Chemother, 48*(12), 4654-4661.

Chanawong, A., M'Zali, F. H., Heritage, J., Lulitanond, A., & Hawkey, P. M. (2001). SHV-12, SHV-5, SHV-2a and VEB-1 extended-spectrum β-lactamases in Gram-negative bacteria isolated in a university hospital in Thailand. *J Antimicrob Chemother, 48*(6), 839-852.

Chaudhari U, B. H., Sharma M. (2008). The Imipenem–EDTA disk method for the rapid identification of metallo β lactamase producing gram negative bacteria. *Indian J Med Res 127*((2)), 406-407.

Claeys, G., Verschraegen, G., de Baere, T., & Vaneechoutte, M. (2000). PER-1 beta-lactamase-producing *Pseudomonas aeruginosa* in an intensive care unit. *J Antimicrob Chemother, 45*(6), 924-925.

Colom, K., Fdz-Aranguiz, A., Suinaga, E., & Cisterna, R. (1995). Emergence of resistance to beta-lactam agents in *Pseudomonas aeruginosa* with group I beta-lactamases in Spain. *Eur J Clin Microbiol Infect Dis, 14*(11), 964-971.

D'Agata, E. M. C. (2004). Rapidly rising prevalence of nosocomial multidrug-resistant, Gram-negative bacilli: a 9-year surveillance study. *Infect Control Hosp Epidemiol, 25*(10), 842-846.

De Champs, C., Rouby, D., Guelon, D., Sirot, J., Sirot, D., Beytout, D.*, et al.* (1991). A case-control study of an outbreak of infections caused by *Klebsiella pneumoniae* strains producing CTX-1 (TEM-3) beta-lactamase. *J Hosp Infect, 18*(1), 5-13.

de Champs, C., Sauvant, M., Chanel, C., Sirot, D., Gazuy, N., & Malhuret, R. (1989). Prospective survey of colonization and infection caused by expanded-spectrum-beta-lactamaseproducing members of the family. *Enterobacteriaceae in an intensive care unit. J Med Microbio, TJ, 2887, 90*.

De Kievit, T. R., Parkins, M. D., Gillis, R. J., Srikumar, R., Ceri, H., Poole, K.*, et al.* (2001). Multidrug efflux pumps: expression patterns and contribution to antibiotic resistance in *Pseudomonas aeruginosa* biofilms. *Antimicrob Agents Chemother, 45*(6), 1761-1770. doi: 10.1128/AAC.45.6.1761-1770.2001.

Defez, C., Fabbro-Peray, P., Bouziges, N., Gouby, A., Mahamat, A., Daures, J.*, et al.* (2004). Risk factors for multidrug-resistant *Pseudomonas aeruginosa* nosocomial infection. *J Hosp Infect, 57*(3), 209-216.

Denning, G. M., Wollenweber, L. A., Railsback, M. A., Cox, C. D., Stoll, L. L., & Britigan, B. E. (1998). *Pseudomonas* pyocyanin increases interleukin-8 expression by human airway epithelial cells. *Infect. Immun, 66*(12), 5777-5784.

Denton, M., Kerr, K., Mooney, L., Keer, V., Rajgopal, A., Brownlee, K.*, et al.* (2002). Transmission of colistin-resistant *Pseudomonas aeruginosa* between patients attending a pediatric cystic fibrosis center. *Pediatr Pulmonol, 34*(4), 257-261.

Doggett, R. G., & Aduan, R. P. (1979). *Pseudomonas aeruginos: clinical manifestations of infection and current therapy*: Academic Press Inc.

Drenkard, E., & Ausubel, F. M. (2002). *Pseudomonas* biofilm formation and antibiotic resistance are linked to phenotypic variation. [10.1038/416740a]. *Nature, 416*(6882), 740-743.

Driscoll, J. A., Brody, S. L., & Kollef, M. H. (2007). The epidemiology, pathogenesis and treatment of *Pseudomonas aeruginosa* infections. *Drugs, 67*(3), 351-368.

Drissi, M., Ahmed, Z. B., Dehecq, B., Bakour, R., Plésiat, P., & Hocquet, D. (2008). Antibiotic susceptibility and mechanisms of beta-lactam resistance among clinical strains of *Pseudomonas aeruginosa*: first report in Algeria *Med Mal Infect, 38*(4), 187-191.

Dubois, V., Arpin, C., Dupart, V., Scavelli, A., Coulange, L., André, C.*, et al.* (2008). β-Lactam and aminoglycoside resistance rates and mechanisms among *Pseudomonas aeruginosa* in French general practice (community and private healthcare centres). *J Antimicrob Chemother, 62*(2), 316-323.

Dumas, J.-L., van Delden, C., Perron, K., & Köhler, T. (2006). Analysis of antibiotic resistance gene expression in *Pseudomonas aeruginosa* by quantitative real-time-PCR. *FEMS Microbiol Lett, 254*(2), 217-225. doi: 10.1111/j.1574-6968.2005.00008.x.

Dupont, H., Mentec, H., Sollet, J., & Bleichner, G. (2001). Impact of appropriateness of initial antibiotic therapy on the outcome of ventilator-associated pneumonia. *Intensive Care Med, 27*(2), 355-362.

Dworkin, M., & Falkow, S. (2006). *The Prokaryotes: Proteobacteria: gamma subclass*: Springer.

El Amin, N., Giske, C., Jalal, S., Keijser, B., Kronvall, G., & Wretlind, B. (2005). Carbapenem resistance mechanisms in *Pseudomonas aeruginosa*: alterations of porin OprD and efflux proteins do not fully explain resistance patterns observed in clinical isolates. *APMIS, 113*, 187 - 196.

Empel, J., Filczak, K., Mrówka, A., Hryniewicz, W., Livermore, D. M., & Gniadkowski, M. (2007). Outbreak of *Pseudomonas aeruginosa* infections with PER-1 extended-spectrum β-lactamase in Warsaw, Poland: further evidence for an international clonal complex. *J Med Microbio, 45*(9), 2829-2834.

Ennis, D. M., & Cobbs, C. G. (1995). The newer cephalosporins. Aztreonam and imipenem. *Infect Dis Clin North Am, 9*(3), 687-713.

Epelman, S., Stack, D., Bell, C., Wong, E., Neely, G. G., Krutzik, S.*, et al.* (2004). Different domains of *Pseudomonas aeruginosa* exoenzyme S activate distinct TLRs. *J Immunol, 173*(3), 2031-2040.

Epp, S. F., Kohler, T., Plesiat, P., Michea-Hamzehpour, M., Frey, J., & Pechere, J. C. (2001). C-terminal region of *Pseudomonas aeruginosa* outer membrane porin OprD modulates susceptibility to meropenem. *Antimicrob Agents Chemother, 45*(6), 1780-1787. doi: 10.1128/AAC.45.6.1780-1787.2001.

Erbay, H., Yalcin, A. N., Serin, S., Turgut, H., Tomatir, E., Cetin, B.*, et al.* (2003). Nosocomial infections in intensive care unit in a Turkish university hospital: a 2-year survey. *Intensive Care Med, 29*(9), 1482-1488.

Evans, K., Adewoye, L., & Poole, K. (2001). MexR repressor of the mexAB-oprM multidrug efflux operon of *Pseudomonas aeruginosa*: identification of MexR binding sites in the mexA-mexR intergenic region. *J Bacteriol, 183*(3), 807-812. doi: 10.1128/JB.183.3.807-812.2001.

Evans, K., & Poole, K. (1999). The MexA-MexB-OprM multidrug efflux system of *Pseudomonas aeruginosa* is growth-phase regulated. *FEMS Microbiol Lett, 173*(1), 35-39. doi: S0378-1097(99)00053-1 [pii].

Falagas, M., Kasiakou, S., Kofteridis, D., Roditakis, G., & Samonis, G. (2006a). Effectiveness and nephrotoxicity of intravenous colistin for treatment of patients with infections due to polymyxin-only-susceptible (POS) gram-negative bacteria. *Eur J Clin Microbiol Infect Dis, 25*(9), 596-599.

Falagas, M. E., Bliziotis, I. A., Kasiakou, S. K., Samonis, G., Athanassopoulou, P., & Michalopoulos, A. (2005a). Outcome of infections due to pandrug-resistant (PDR) Gram-negative bacteria. *BMC Infect Dis, 5*, 24. doi: 1471-2334-5-24 [pii] 10.1186/1471-2334-5-24.

Falagas, M. E., Fragoulis, K. N., Kasiakou, S. K., Sermaidis, G. J., & Michalopoulos, A. (2005b). Nephrotoxicity of intravenous colistin: a prospective evaluation. *Int J Antimicrob Agents, 26*(6), 504-507.

Falagas, M. E., & Kasiakou, S. K. (2006). Use of international units when dosing colistin will help decrease confusion related to various formulations of the drug around the world. *Antimicrob Agents Chemother, 50*(6), 2274-2275.

Falagas, M. E., Kasiakou, S. K., & Saravolatz, L. D. (2005c). Colistin: the revival of polymyxins for the management of multidrug-resistant gram-negative bacterial infections. *Clin Infect Dis, 40*(9), 1333.

Falagas, M. E., Kastoris, A. C., Kapaskelis, A. M., & Karageorgopoulos, D. E. (2010). Fosfomycin for the treatment of multidrug-resistant, including extended-spectrum [beta]-lactamase producing, Enterobacteriaceae infections: a systematic review. *Lancet Infect Dis, 10*(1), 43-50.

Falagas, M. E., Kastoris, A. C., Karageorgopoulos, D. E., & Rafailidis, P. I. (2009). Fosfomycin for the treatment of infections caused by multidrug-resistant non-fermenting Gram-negative bacilli: a systematic review of

microbiological, animal and clinical studies. *Int J Antimicrob Agents, 34*(2), 111-120.

Falagas, M. E., Koletsi, P. K., Kopterides, P., & Michalopoulos, A. (2006b). Risk factors for isolation of strains susceptible only to polymyxin among patients with *Pseudomonas aeruginosa* bacteremia. *Antimicrob Agents Chemother, 50*(7), 2541-2543.

Farra, A., Islam, S., Strålfors, A., Sörberg, M., & Wretlind, B. (2008). Role of outer membrane protein OprD and penicillin-binding proteins in resistance of *Pseudomonas aeruginosa* to imipenem and meropenem. *Int J Antimicrob Agents 31*(5), 427-433. doi: DOI: 10.1016/j.ijantimicag.2007.12.016.

Feldman, M., Bryan, R., Rajan, S., Scheffler, L., Brunnert, S., Tang, H., *et al.* (1998). Role of flagella in pathogenesis of *Pseudomonas aeruginosa* pulmonary infection. *Infect Immun, 66*(1), 43-51.

Fernández-Cuenca, F., Martínez-Martínez, L., Conejo, M. C., Ayala, J. A., Perea, E. J., & Pascual, A. (2003). Relationship between β-lactamase production, outer membrane protein and penicillin-binding protein profiles on the activity of carbapenems against clinical isolates of Acinetobacter baumannii. *J Antimicrob Chemother, 51*(3), 565-574. doi: 10.1093/jac/dkg097.

Finch, R., Pritchard, D., Bycroft, B., Williams, P., & Stewart, G. (1998). Quorum sensing: a novel target for anti-infective therapy. *J Antimicrob Chemother 42*(5), 569-571.

Fish, D. N., Piscitelli, S. C., & Danziger, L. H. (1995). Development of resistance during antimicrobial therapy: a review of antibiotic classes and patient characteristics in 173 studies. *Pharmacotherapy: Pharmacotherapy, 15*(3), 279-291.

Fisher, J. F., Meroueh, S. O., & Mobashery, S. (2005). Bacterial Resistance to β-Lactam Antibiotics: Compelling Opportunism, Compelling Opportunity†. *Chem Rev, 105*(2), 395-424. doi: 10.1021/cr030102i.

Flamm, R. K., Weaver, M. K., Thornsberry, C., Jones, M. E., Karlowsky, J. A., & Sahm, D. F. (2004). Factors associated with relative rates of antibiotic resistance in *Pseudomonas aeruginosa* isolates tested in clinical laboratories in the United States from 1999 to 2002. *Antimicrob Agents Chemother, 48*(7), 2431-2436.

Fluit, A. C., & Schmitz, F. J. (1999). Class 1 integrons, gene cassettes, mobility, and epidemiology. *Eur J Clin Microbiol Infect Dis, 18*(11), 761-770.

Fridkin, S. K., Hill, H. A., Volkova, N. V., Edwards, J. R., Lawton, R. M., Gaynes, R. P., *et al.* (2002). Temporal changes in prevalence of antimicrobial resistance in 23 US hospitals. *Emerging Infec Dis, 8*(7), 697.

Furtado, G. H. C., d'Azevedo, P. A., Santos, A. F., Gales, A. C., Pignatari, A. C. C., & Medeiros, E. A. S. (2007). Intravenous polymyxin B for the treatment of

nosocomial pneumonia caused by multidrug-resistant *Pseudomonas aeruginosa*. *Int J Antimicrob Agents, 30*(4), 315-319.

Galani, I., Xirouchaki, E., Kanellakopoulou, K., Petrikkos, G., & Giamarellou, H. (2002). Transferable plasmid mediating resistance to multiple antimicrobial agents in *Klebsiella pneumoniae* isolates in Greece. *Clin Microbiol Infect, 8*(9), 579-588. doi: 391 [pii].

Garnacho-Montero, J., Sa-Borges, M., Sole-Violan, J., Barcenilla, F., Escoresca-Ortega, A., Ochoa, M.*, et al.* (2007). Optimal management therapy for *Pseudomonas aeruginosa* ventilator-associated pneumonia: An observational, multicenter study comparing monotherapy with combination antibiotic therapy*. *Crit Care Med, 35*(8), 1888.

Gasink, L. B., Fishman, N. O., Weiner, M. G., Nachamkin, I., Bilker, W. B., & Lautenbach, E. (2006). Fluoroquinolone-resistant *Pseudomonas aeruginosa*: assessment of risk factors and clinical impact. *Am J Med, 119*(6), 526. e519-526. e525.

Gauthier, A., Thomas, N. A., & Finlay, B. B. (2003). Bacterial injection machines. *J Biol Chem, 278*(28), 25273.

Georges, B., Conil, J. M., Dubouix, A., Archambaud, M., Bonnet, E., Saivin, S.*, et al.* (2006). Risk of emergence of *Pseudomonas aeruginosa* resistance to [beta]-lactam antibiotics in intensive care units. *Crit Care Med, 34*(6), 1636.

Gessard, C. (1984). On the blue and green coloration that appears on bandages. *Review of Clin Infect Dis, 6*(Supplement 3), S775-S776.

Ghuysen, J. M. (1994). Molecular structures of penicillin-binding proteins and beta-lactamases. *Trends Microbiol, 2*(10), 372-380.

Giamarellos-Bourboulis, E., Grecka, P., & Giamarellou, H. (1996). In-vitro interactions of DX-8739, a new carbapenem, meropenem and imipenem with amikacin against multiresistant *Pseudomonas aeruginosa*. *J Antimicrob Chemother, 38*(2), 287-291.

Giamarellos-Bourboulis, E., Sambatakou, H., Galani, I., & Giamarellou, H. (2003). In vitro interaction of colistin and rifampin on multidrug-resistant Pseudomonas aeruginosa. *J Chemother, 15*(3), 235-238.

Giamarellos-Bourboulis, E. J., Kentepozidis, N., Antonopoulou, A., Plachouras, D., Tsaganos, T., & Giamarellou, H. (2005). Postantibiotic effect of antimicrobial combinations on multidrug-resistant *Pseudomonas aeruginosa*. *Diagn Microbiol Infect Dis, 51*(2), 113-117.

Giamarellou, H. (2002). Prescribing guidelines for severe *Pseudomonas* infections. *J Antimicrob Chemother, 49*(2), 229-233.

Giamarellou, H., & Kanellakopoulou, K. (2008). Current therapies for Pseudomonas aeruginosa. *Crit Care Clin, 24*(2), 261-278.

Giamarellou, H., Zissis, N. P., Tagari, G., & Bouzos, J. (1984). In vitro synergistic activities of aminoglycosides and new beta-lactams against multiresistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 25*(4), 534-536.

Gibb, A. P., Tribuddharat, C., Moore, R. A., Louie, T. J., Krulicki, W., Livermore, D. M.*, et al.* (2002). Nosocomial outbreak of carbapenem-resistant *Pseudomonas aeruginosa* with a new bla(IMP) allele, bla(IMP-7). *Antimicrob Agents Chemother, 46*(1), 255-258.

Girlich, D., Naas, T., Leelaporn, A., Poirel, L., Fennewald, M., & Nordmann, P. (2002). Nosocomial spread of the integron-located veb-1-like cassette encoding an extended-pectrum beta-lactamase in *Pseudomonas aeruginosa* in Thailand. *Clin Infect Dis, 34*(5), 603-611. doi: CID010685 [pii] 10.1086/338786.

Giske, C. G., Boren, C., Wretlind, B., & Kronvall, G. (2005). Meropenem susceptibility breakpoint for *Pseudomonas aeruginosa* strains hyperproducing mexB mRNA. *Clin Microbiol Infect, 11*(8), 662-669. doi: CLM1182 [pii] 10.1111/j.1469-0691.2005.01182.x.

Giske, C. G., Buaro, L., Sundsfjord, A., & Wretlind, B. (2008a). Alterations of porin, pumps, and penicillin-binding proteins in carbapenem resistant clinical isolates of *Pseudomonas aeruginosa*. *Microb Drug Resist, 14*(1), 23-30. doi: 10.1089/mdr.2008.0778.

Giske, C. G., Monnet, D. L., Cars, O., & Carmeli, Y. (2008b). Clinical and economic impact of common multidrug-resistant gram-negative bacilli. *Antimicrob Agents Chemother, 52*(3), 813-821. doi: AAC.01169-07 [pii] 10.1128/AAC.01169-07.

Gniadkowski, M. (2001). Evolution and epidemiology of extended-spectrum β-lactamases (ESBLs) and ESBL-producing microorganisms. *Clin Microbiol Infect, 7*(11), 597-608.

Gori, A., Espinasse, F., Deplano, A., Nonhoff, C., Nicolas, M. H., & Struelens, M. J. (1996). Comparison of pulsed-field gel electrophoresis and randomly amplified DNA polymorphism analysis for typing extended-spectrum-beta-lactamase-producing Klebsiella pneumoniae. *Clin Microbiol Infect, 34*(10), 2448-2453.

Gotoh, N., Tsujimoto, H., Nomura, A., Okamoto, K., Tsuda, M., & Nishino, T. (1998a). Functional replacement of OprJ by OprM in the MexCD-OprJ multidrug efflux system of *Pseudomonas aeruginosa*. *FEMS Microbiol Lett, 165*(1), 21-27. doi: S0378-1097(98)00250-X [pii].

Gotoh, N., Tsujimoto, H., Tsuda, M., Okamoto, K., Nomura, A., Wada, T.*, et al.* (1998b). Characterization of the MexC-MexD-OprJ multidrug efflux system in DeltamexA-mexB-oprM mutants of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 42*(8), 1938-1943.

Gupta, V., Datta, P., & Chander, J. (2006). Prevalence of metallo-[beta] lactamase (MBL) producing *Pseudomonas spp*. and *Acinetobacter spp*. in a tertiary care hospital in India. *J. Infect, 52*(5), 311-314.

Gutierrez, O., Juan, C., Cercenado, E., Navarro, F., Bouza, E., Coll, P.*, et al.* (2007). Molecular Epidemiology and Mechanisms of Carbapenem Resistance in *Pseudomonas aeruginosa* Isolates from Spanish Hospitals. *Antimicrob. Agents Chemother., 51*(12), 4329-4335. doi: 10.1128/aac.00810-07.

Hachem, R. Y., Chemaly, R. F., Ahmar, C. A., Jiang, Y., Boktour, M. R., Rjaili, G. A.*, et al.* (2007). Colistin is effective in treatment of infections caused by multidrug-resistant *Pseudomonas aeruginosa* in cancer patients. *Antimicrob. Agents Chemother, 51*(6), 1905-1911.

Hancock, R. E., & Brinkman, F. S. (2002). Function of pseudomonas porins in uptake and efflux. *Annu Rev Microbiol, 56*, 17-38. doi: 10.1146/annurev.micro.56.012302.160310012302.160310 [pii].

Hancock, R. E., Siehnel, R., & Martin, N. (1990). Outer membrane proteins of Pseudomonas. *Mol Microbiol, 4*(7), 1069-1075.

Hancock, R. E. W. (1998). Resistance mechanisms in *Pseudomonas aeruginosa* and other nonfermentative gram-negative bacteria. *Clin Infect Dis, 27*(Supplement 1), S93.

Hanson, N. D., & Sanders, C. C. (1999). Regulation of inducible AmpC beta-lactamase expression among Enterobacteriaceae. *Curr Pharm Des, 5*(11), 881-894.

Hauser, A. R., Cobb, E., Bodí, M., Mariscal, D., Vallés, J., Engel, J. N.*, et al.* (2002). Type III protein secretion is associated with poor clinical outcomes in patients with ventilator-associated pneumonia caused by *Pseudomonas aeruginosa*. *Crit Care Med, 30*(3), 521.

Hauser, A. R., Fleiszig, S., Kang, P. J., Mostov, K., & Engel, J. N. (1998). Defects in type III secretion correlate with internalization of *Pseudomonas aeruginosa* by epithelial cells. *Infect Immun, 66*(4), 1413-1420.

Hauser, A. R., & Sriram, P. (2005). Severe *Pseudomonas aeruginosa* infections. Tackling the conundrum of drug resistance. *Postgrad Med, 117*(1), 41-48.

Häußler, S., Ziegler, I., Löttel, A., Götz, F. v., Rohde, M., Wehmhöhner, D.*, et al.* (2003). Highly adherent small-colony variants of *Pseudomonas aeruginosa* in cystic fibrosis lung infection. *J Med Microbiol, 52*(4), 295-301. doi: 10.1099/jmm.0.05069-0.

Hemalatha, V., Sekar, U., & Kamat, V. (2005). Detection of metallo betalactamase producing *Pseudomonas aeruginosa* in hospitalized patients. *Indian J Med Res, 122*(2), 148-152.

Henrichfreise, B., Wiegand, I., Pfister, W., & Wiedemann, B. (2007). Resistance Mechanisms of Multiresistant *Pseudomonas aeruginosa* Strains from Germany and Correlation with Hypermutation. *Antimicrob. Agents Chemother., 51*(11), 4062-4070. doi: 10.1128/aac.00148-07.

Hentzer, M., Eberl, L., Nielsen, J., & Givskov, M. (2003). Quorum sensing: a novel target for the treatment of biofilm infections. *BioDrugs, 17*(4), 241-250.

Hirakata, Y., Izumikawa, K., Yamaguchi, T., Takemura, H., Tanaka, H., Yoshida, R.*, et al.* (1998). Rapid Detection and Evaluation of Clinical Characteristics of Emerging Multiple-Drug-Resistant Gram-Negative Rods Carrying the Metallo-β-Lactamase Genebla IMP. *Antimicrob. Agents Chemother, 42*(8), 2006-2011.

Ho, S. E., Subramaniam, G., Palasubramaniam, S., & Navaratnam, P. (2002). Carbapenem-resistant *Pseudomonas aeruginosa* in malaysia producing IMP-7 beta-lactamase. *Antimicrob Agents Chemother, 46*(10), 3286-3287.

Hocquet, D., Muller, A., Blanc, K., Plesiat, P., Talon, D., Monnet, D.*, et al.* (2008). Relationship between antibiotic use and incidence of MexXY-OprM overproducers among clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 52*, 1173 - 1175.

Hocquet, D., Nordmann, P., El Garch, F., Cabanne, L., & Plesiat, P. (2006). Involvement of the MexXY-OprM Efflux System in Emergence of Cefepime Resistance in Clinical Strains of *Pseudomonas aeruginosa*. *Antimicrob. Agents Chemother., 50*(4), 1347-1351. doi: 10.1128/aac.50.4.1347-1351.2006.

Hocquet, D., Roussel-Delvallez, M., Cavallo, J., & Plesiat, P. (2007). MexAB-OprM- and MexXY-overproducing mutants are very prevalent among clinical strains of *Pseudomonas aeruginosa* with reduced susceptibility to ticarcillin. *Antimicrob Agents Chemother, 51*, 1582 - 1583.

Hocquet, D., Vogne, C., El Garch, F., Vejux, A., Gotoh, N., Lee, A.*, et al.* (2003). MexXY-OprM Efflux Pump Is Necessary for Adaptive Resistance of *Pseudomonas aeruginosa* to Aminoglycosides. *Antimicrob. Agents Chemother., 47*(4), 1371-1375. doi: 10.1128/aac.47.4.1371-1375.2003.

Hooper, D. C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerg Infect Dis, 7*(2), 337-341.

Horianopoulou, M., Lambropoulos, S., Papafragas, E., & Falagas, M. (2005). Effect of aerosolized colistin on multidrug-resistant *Pseudomonas aeruginosa* in bronchial secretions of patients without cystic fibrosis. *J Chemother, 17*(5), 536-538.

Huang, H., Jeanteur, D., Pattus, F., & Hancock, R. E. (1995). Membrane topology and site-specific mutagenesis of *Pseudomonas aeruginosa* porin OprD . *Mol Microbiol, 16*(5), 931-941.

Huang, Y. T., Chang, S. C., Lauderdale, T. L., Yang, A. J., & Wang, J. T. (2007). Molecular epidemiology of carbapenem-resistant *Pseudomonas aeruginosa* carrying metallo-beta-lactamase genes in Taiwan. *Diagn Microbiol Infect Dis, 59*(2), 211-216. doi: S0732-8893(07)00033-8 [pii] 10.1016/j.diagmicrobio.2007.01.009.

Hughes, V. M., & Datta, N. (1983). Conjugative plasmids in bacteria of the 'pre-antibiotic'era.

Humphreys, H. (2009). Preventing surgical site infection. Where now? *J Hosp Infect, 73*(4), 316-322.

Hwa, W. E., Subramaniam, G., Navaratnam, P., & Sekaran, S. D. (2009). Detection and characterization of class 1 integrons among carbapenem-resistant isolates of *Acinetobacter spp*. in Malaysia. *J Microbiol Immunol Infect, 42*(1), 54-62.

Ishii, Y., Ohno, A., Taguchi, H., Imajo, S., Ishiguro, M., & Matsuzawa, H. (1995). Cloning and sequence of the gene encoding a cefotaxime-hydrolyzing class A beta-lactamase isolated from Escherichia coli. *Antimicrob Agents Chemother, 39*(10), 2269-2275.

Islam, S., Jalal, S., & Wretlind, B. (2004). Expression of the MexXY efflux pump in amikacin-resistant isolates of *Pseudomonas aeruginosa*. *Clin Microbiol Infect, 10*(10), 877-883. doi: 10.1111/j.1469-0691.2004.00991.x.

Jacoby, G. A. (1997). Extended-spectrum [beta]-lactamases and other enzymes providing resistance to oxyimino-[beta]-lactams. *Infect Dis Clin North Am, 11*(4), 875-887.

Jacoby, G. A. (2009). AmpC beta-lactamases. *Clin Microbiol Rev, 22*(1), 161-182, Table of Contents. doi: 22/1/161 [pii]10.1128/CMR.00036-08.

Jacoby, G. A., & Munoz-Price, L. S. (2005). The new β-lactamases. *New England Journal of Medicine, 352*(4), 380-391.

Jayakumar, S., & Appalaraju, B. (2007). Prevalence of multi and pan drug resistant *Pseudomonas aeruginosa* with respect to ESBL and MBL in a tertiary care hospital. *Indian J Pathol Microbiol, 50*(4), 922-925.

Jeannot, K., Sobel, M., El Garch, F., Poole, K., & Plesiat, P. (2005). Induction of the MexXY efflux pump in *Pseudomonas aeruginosa* is dependent on drug-ribosome interaction. *J Bacteriol, 187*, 5341 - 5346.

Jiang, X., Zhang, Z., Li, M., Zhou, D., Ruan, F., & Lu, Y. (2006). Detection of extended-spectrum beta-lactamases in clinical isolates of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 50*(9), 2990-2995. doi: 50/9/2990 [pii]10.1128/AAC.01511-05.

Jo, J. T., Brinkman, F. S., & Hancock, R. E. (2003). Aminoglycoside efflux in *Pseudomonas aeruginosa*: involvement of novel outer membrane proteins. *Antimicrob Agents Chemother, 47*(3), 1101-1111.

Join-Lambert, O. F., Michéa-Hamzehpour, M., Köhler, T., Chau, F., Faurisson, F., Dautrey, S., *et al.* (2001). Differential Selection of Multidrug Efflux Mutants by Trovafloxacin and Ciprofloxacin in an Experimental Model of *Pseudomonas aeruginosa* Acute Pneumonia in Rats. *Antimicrob Agents Chemother, 45*(2), 571-576. doi: 10.1128/aac.45.2.571-576.2001.

Jombo, G., Akpan, S., Epoke, J., Denen, A., & Odey, F. (2010). Multidrug resistant *Psudomonas aeruginosa* infections complicating surgical wounds and the potential challenges in managing post-operative wound infections: University of Calabar Teaching Hospital experience. *Asian Pac J Trop Med, 3*(6), 479-482.

Jones, M. E., Draghi, D. C., Thornsberry, C., Karlowsky, J. A., Sahm, D. F., & Wenzel, R. P. (2004a). Emerging resistance among bacterial pathogens in the intensive care unit--a European and North American Surveillance study (2000-2002). *Ann Clin Microbiol Antimicrob, 3*, 14. doi: 10.1186/1476-0711-3-141476-0711-3-14 [pii].

Jones, R. N. (1998). Important and emerging β-lactamase-mediated resistances in hospital-based pathogens: the Amp C enzymes. *Diagn Microbiol Infect Dis, 31*(3), 461-466.

Jones, R. N., Huynh, H. K., Biedenbach, D. J., Fritsche, T. R., & Sader, H. S. (2004b). Doripenem (S-4661), a novel carbapenem: comparative activity against contemporary pathogens including bactericidal action and preliminary in vitro methods evaluations. *J Antimicrob Chemother, 54*(1), 144-154.

Jones, R. N., Kirby, J. T., Beach, M. L., Biedenbach, D. J., & Pfaller, M. A. (2002). Geographic variations in activity of broad-spectrum β-lactams against *Pseudomonas aeruginosa*: summary of the worldwide SENTRY Antimicrobial Surveillance Program (1997–2000). *Diagn Microbiol Infect Dis, 43*(3), 239-243.

Juan, C., Moyá, B., Pérez, J. L., & Oliver, A. (2006). Stepwise Up-regulation of the *Pseudomonas aeruginosa* Chromosomal Cephalosporinase Conferring High-Level β-Lactam Resistance Involves Three AmpD Homologues. *Antimicrob Agents Chemother, 50*(5), 1780-1787. doi: 10.1128/aac.50.5.1780-1787.2006.

Jung, R., Fish, D., Obritsch, M., & MacLaren, R. (2004). Surveillance of multi-drug resistant Pseudomonas aeruginosa in an urban tertiary-care teaching hospital. *J Hosp Infect, 57*(2), 105-111.

Kallel, H., Hergafi, L., Bahloul, M., Hakim, A., Dammak, H., Chelly, H., *et al.* (2007). Safety and efficacy of colistin compared with imipenem in the treatmentof ventilator-associated pneumonia: a matched case–control study. *Intensive Care Med, 33*(7), 1162-1167.

Kallel, H., Mahjoubi, F., Dammak, H., Bahloul, M., Hamida, C. B., Chelly, H., *et al.* (2008). Correlation between antibiotic use and changes in susceptibility

patterns of *Pseudomonas aeruginosa* in a medical-surgical intensive care unit. *Indian journal of critical care medicine: Indian J Crit Care Med, 12*(1), 18.

Kang, H. Y., Jeong, Y. S., Oh, J. Y., Tae, S. H., Choi, C. H., Moon, D. C., *et al.* (2005). Characterization of antimicrobial resistance and class 1 integrons found in *Escherichia coli* isolates from humans and animals in Korea. *J Antimicrob Chemother, 55*(5), 639-644. doi: dki076 [pii] 10.1093/jac/dki076.

Kanj, S. S., & Kanafani, Z. A. (2011). Current Concepts in Antimicrobial Therapy Against Resistant Gram-Negative Organisms: Extended-Spectrum β-Lactamase–Producing Enterobacteriaceae, Carbapenem-Resistant Enterobacteriaceae, and Multidrug-Resistant *Pseudomonas aeruginosa*. .

Karim, A., Poirel, L., Nagarajan, S., & Nordmann, P. (2001). Plasmid-mediated extended-spectrum β-lactamase (CTX-M-3 like) from India and gene association with insertion sequence ISEcp1. *FEMS Microbiol Lett, 201*(2), 237-241.

Karthikeyan, K., Thirunarayan, M. A., & Krishnan, P. (2010). Coexistence of blaOXA-23 with *blaNDM-1* and armA in clinical isolates of Acinetobacter baumannii from India. *J Antimicrob Chemother, 65*(10), 2253-2254. doi: dkq273 [pii]10.1093/jac/dkq273.

Kasiakou, S. K., Michalopoulos, A., Soteriades, E. S., Samonis, G., Sermaides, G. J., & Falagas, M. E. (2005). Combination therapy with intravenous colistin for management of infections due to multidrug-resistant Gram-negative bacteria in patients without cystic fibrosis. *Antimicrob Agents Chemother, 49*(8), 3136-3146.

Kato, J., Kim, H.-E., Takiguchi, N., Kuroda, A., & Ohtake, H. (2008). *Pseudomonas aeruginosa* as a model microorganism for investigation of chemotactic behaviors in ecosystem. *J Biosci Bioeng, 106*(1), 1-7. doi: 10.1263/jbb.106.1.

Kaufmann, G. F., Park, J., & Janda, K. D. (2008). Bacterial quorum sensing: a new target for anti-infective immunotherapy.

Khosravi, Y., Tee Tay, S., & Vadivelu, J. (2010). Metallo-[beta]-lactamase-producing imipenem-resistant *Pseudomonas aeruginosa* clinical isolates in a university teaching hospital in Malaysia: detection of IMP-7 and first identification of IMP-4, VIM-2, and VIM-11. [doi: DOI: 10.1016/j.diagmicrobio.2010.02.010]. *Diagn Microbiol Infect Dis, 67*(3), 294-296.

King EO, Ward MK, Raney DE (1954). "Two simple media for the demonstration of pyocyanin and fluorescein." *J Lab Clin Med,* 44 (2), 301–7. PMID 13184240.

Kiser, T. H., Obritsch, M. D., Jung, R., MacLaren, R., & Fish, D. N. (2010). Efflux pump contribution to multidrug resistance in clinical isolates of Pseudomonas aeruginosa. *Pharmacotherapy, 30*(7), 632-638. doi: 10.1592/phco.30.7.63210.1592/phco.30.7.632 [pii].

Kliebe, C., Nies, B. A., Meyer, J. F., Tolxdorff-Neutzling, R. M., & Wiedemann, B. (1985). Evolution of plasmid-coded resistance to broad-spectrum cephalosporins. *Antimicrob Agents Chemother, 28*(2), 302-307.

Ko¨hler, T., Michéa-Hamzehpour, M., Henze, U., Gotoh, N., Kocjancic Curty, L., & Pechère, J.-C. (1997). Characterization of MexE–MexF–OprN, a positively regulated multidrug efflux system of Pseudomonas aeruginosa. *Mol Microbiol, 23*(2), 345-354. doi: 10.1046/j.1365-2958.1997.2281594.x.

Koh, T. H., Wang, G. C., & Sng, L. H. (2004). IMP-1 and a novel metallo-beta-lactamase, VIM-6, in fluorescent pseudomonads isolated in Singapore. *Antimicrob Agents Chemother, 48*(6), 2334-2336. doi: 10.1128/AAC.48.6.2334-2336.200448/6/2334 [pii].

Kohler, T., Curty, L. K., Barja, F., van Delden, C., & Pechere, J.-C. (2000). Swarming of *Pseudomonas aeruginosa* Is Dependent on Cell-to-Cell Signaling and Requires Flagella and Pili. *J. Bacteriol., 182*(21), 5990-5996. doi: 10.1128/jb.182.21.5990-5996.2000.

Kohler, T., Epp, S. F., Curty, L. K., & Pechere, J. C. (1999a). Characterization of MexT, the regulator of the MexE-MexF-OprN multidrug efflux system of Pseudomonas aeruginosa. *J Bacteriol, 181*(20), 6300-6305.

Kohler, T., Kok, M., Michea-Hamzehpour, M., Plesiat, P., Gotoh, N., Nishino, T., *et al.* (1996). Multidrug efflux in intrinsic resistance to trimethoprim and sulfamethoxazole in Pseudomonas aeruginosa. *Antimicrob Agents Chemother, 40*(10), 2288-2290.

Kohler, T., Michea-Hamzehpour, M., Epp, S. F., & Pechere, J. C. (1999b). Carbapenem activities against *Pseudomonas aeruginosa*: respective contributions of OprD and efflux systems. *Antimicrob Agents Chemother, 43*(2), 424-427.

Kohler, T., Pechere, J. C., & Plesiat, P. (1999c). Bacterial antibiotic efflux systems of medical importance. *Cell Mol Life Sci, 56*(9-10), 771-778.

Kolayli, F., Gacar, G., Karadenizli, A., Sanic, A., & Vahaboglu, H. (2005). PER-1 is still widespread in Turkish hospitals among *Pseudomonas aeruginosa* and Acinetobacter spp. *FEMS Microbiol Lett, 249*(2), 241-245.

Kollef, M. H. (2006). Providing appropriate antimicrobial therapy in the intensive care unit: Surveillance vs. de-escalation*. *Crit Care Med, 34*(3), 903.

Kuehn, B. M. (2011). Proposals seek to reduce resistance, boost development of new antibiotics. *JAMA, 305*(18), 1845.

Kumarasamy, K. K., Toleman, M. A., Walsh, T. R., Bagaria, J., Butt, F., Balakrishnan, R., *et al.* (2010). Emergence of a new antibiotic resistance mechanism in India, Pakistan, and the UK: a molecular, biological, and epidemiological study. *Lancet Infect Dis, 10*(9), 597-602. doi: 10.1016/s1473-3099(10)70143-2.

Lagatolla, C., Edalucci, E., Dolzani, L., Riccio, M. L., De Luca, F., Medessi, E., *et al.* (2006). Molecular evolution of metallo-beta-lactamase-producing *Pseudomonas aeruginosa* in a nosocomial setting of high-level endemicity. *J Clin Microbiol, 44*(7), 2348-2353. doi: 44/7/2348 [pii]10.1128/JCM.00258-06.

Lagatolla, C., Tonin, E. A., Monti-Bragadin, C., Dolzani, L., Gombac, F., Bearzi, C., *et al.* (2004). Endemic carbapenem-resistant Pseudomonas aeruginosa with acquired metallo-beta-lactamase determinants in European hospital. *Emerg Infect Dis, 10*(3), 535-538.

Lalitha, M. K., Manayani, D. J., Priya, L., Jesudason, M. V., Thomas, K., & Steinhoff, M. C. (1997). E test as an alternative to conventional MIC determination for surveillance of drug resistant Streptococcus pneumoniae. *Indian J Med Res, 106*, 500-503.

Lambert, P. (2002). Mechanisms of antibiotic resistance in *Pseudomonas aeruginosa*. *J R Soc Med, 95*(Suppl 41), 22.

Landsperger, W., Kelly-Wintenberg, K., Montie, T., Knight, L., Hansen, M., Huntenburg, C., *et al.* (1994). Inhibition of bacterial motility with human antiflagellar monoclonal antibodies attenuates Pseudomonas aeruginosa-induced pneumonia in the immunocompetent rat. *Infect Immun, 62*(11), 4825-4830.

Lau, G. W., Ran, H., Kong, F., Hassett, D. J., & Mavrodi, D. (2004a). Pseudomonas aeruginosa Pyocyanin Is Critical for Lung Infection in. Mice. *Infect Immun, 72*(7), 4275-4278. doi: 10.1128/iai.72.7.4275-4278.2004

Lau, G. W., Ran, H., Kong, F., Hassett, D. J., & Mavrodi, D. (2004b). Pseudomonas aeruginosa pyocyanin is critical for lung infection in mice. *Infect Immun, 72*(7), 4275-4278.

Lautenbach, E., Patel, J. B., Bilker, W. B., Edelstein, P. H., & Fishman, N. O. (2001). Extended-spectrum beta-lactamase-producing Escherichia coli and *Klebsiella pneumoniae*: risk factors for infection and impact of resistance on outcomes. *Clin Infect Dis, 32*(8), 1162-1171. doi: CID000853 [pii]10.1086/319757.

Lee, K., Ha, G. Y., Shin, B. M., Kim, J. J., Kang, J. O., Jang, S. J., *et al.* (2004). Metallo-[beta]-lactamase-producing Gram-negative bacilli in Korean Nationwide Surveillance of Antimicrobial Resistance group hospitals in 2003: Continued prevalence of VIM-producing pseudomonas spp. and increase of IMP-producing *Acinetobacter spp*. *Diagn Microbiol Infect Dis, 50*(1), 51-58.

Lee, S., Park, Y. J., Kim, M., Lee, H. K., Han, K., Kang, C. S., *et al.* (2005). Prevalence of Ambler class A and D beta-lactamases among clinical isolates of Pseudomonas aeruginosa in Korea. *J Antimicrob Chemother, 56*(1), 122-127. doi: dki160 [pii]10.1093/jac/dki160.

Lee, S. H., Kim, J. Y., Shin, S. H., An, Y. J., Choi, Y. W., Jung, Y. C., *et al.* (2003). Dissemination of SHV-12 and characterization of new AmpC-type beta-lactamase genes among clinical isolates of Enterobacter species in Korea. *J Med Microbio, 41*(6), 2477-2482.

Legaree, B. A., Daniels, K., Weadge, J. T., Cockburn, D., & Clarke, A. J. (2007). Function of penicillin-binding protein 2 in viability and morphology of Pseudomonas aeruginosa. *J Antimicrob Chemother, 59*(3), 411-424. doi: dkl536 [pii]10.1093/jac/dkl536.

Leidal, K. G., Munson, K. L., & Denning, G. M. (2001). Small molecular weight secretory factors from *Pseudomonas aeruginosa* have opposite effects on IL-8 and RANTES expression by human airway epithelial cells. *Am. J. Respir Cell Mo. Biol, 25*(2), 186-195

.

Lepper, P. M., Grusa, E., Reichl, H., Högel, J., & Trautmann, M. (2002). Consumption of imipenem correlates with β-lactam resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 46*(9), 2920-2925.

Li, X.-Z., & Nikaido, H. (2009). Efflux-Mediated Drug Resistance in Bacteria: An Update. *Drugs, 69*(12), 1555-1623.

Li, X. Z., Barre, N., & Poole, K. (2000a). Influence of the MexA-MexB-oprM multidrug efflux system on expression of the MexC-MexD-oprJ and MexE-MexF-oprN multidrug efflux systems in *Pseudomonas aeruginosa*. *J Antimicrob Chemother, 46*(6), 885-893.

Li, X. Z., Livermore, D. M., & Nikaido, H. (1994). Role of efflux pump (s) in intrinsic resistance of *Pseudomonas aeruginosa*: resistance to tetracycline, chloramphenicol, and norfloxacin. *Antimicrob Agents Chemother, 38*(8), 1732-1741.

Li, X. Z., Nikaido, H., & Poole, K. (1995). Role of mexA-mexB-oprM in antibiotic efflux in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 39*(9), 1948-1953.

Li, X. Z., & Poole, K. (1999). Organic solvent-tolerant mutants of Pseudomonas aeruginosa display multiple antibiotic resistance. *Can J Microbiol, 45*(1), 18-22.

Li, X. Z., Poole, K., & Nikaido, H. (2003a). Contributions of MexAB-OprM and an EmrE homolog to intrinsic resistance of *Pseudomonas aeruginosa* to aminoglycosides and dyes. *Antimicrob Agents Chemother, 47*(1), 27-33.

Li, X. Z., Zhang, L., & Poole, K. (1998a). Role of the Multidrug Efflux Systems of Pseudomonas aeruginosa in Organic Solvent Tolerance. *J Bacteriol, 180*(11), 2987-2991.

Li, X. Z., Zhang, L., & Poole, K. (2000b). Interplay between the MexA-MexB-OprM multidrug efflux system and the outer membrane barrier in the multiple antibiotic resistance of *Pseudomonas aeruginosa*. *J Antimicrob Chemother, 45*(4), 433-436.

Li, X. Z., Zhang, L., Srikumar, R., & Poole, K. (1998b). Beta-lactamase inhibitors are substrates for the multidrug efflux pumps of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 42*(2), 399-403.

Li, Y., Mima, T., Komori, Y., Morita, Y., Kuroda, T., Mizushima, T.*, et al.* (2003b). A new member of the tripartite multidrug efflux pumps, MexVW-OprM, in *Pseudomonas aeruginosa*. *J Antimicrob Chemother, 52*(4), 572-575. doi: 10.1093/jac/dkg390dkg390 [pii].

Lim, K. T., Yasin, R. M., Yeo, C. C., Puthucheary, S. D., Balan, G., Maning, N.*, et al.* (2009). Genetic fingerprinting and antimicrobial susceptibility profiles of *Pseudomonas aeruginosa* hospital isolates in Malaysia. *J Microbiol Immunol Infect., 42*(3), 197.

Lim, W. S., van der Eerden, M. M., Laing, R., Boersma, W. G., Karalus, N., Town, G. I.*, et al.* (2003). Defining community acquired pneumonia severity on presentation to hospital: an international derivation and validation study. *Thorax, 58*(5), 377-382. doi: 10.1136/thorax.58.5.377

Lister, P. D. (2000). β-Lactamase Inhibitor Combinations with Extended-Spectrum Penicillins: Factors Influencing Antibacterial Activity against *Enterobacteriaceae* and *Pseudomonas aeruginosa*. *Pharmacotherapy: The Pharmacotherapy, 20*(9P2), 213S-218S.

Lister, P. D., & Wolter, D. J. (2005). Levofloxacin-imipenem combination prevents the emergence of resistance among clinical isolates of *Pseudomonas aeruginosa*. *Clin Infect Dis, 40*(Supplement 2), S105.

Lister, P. D., Wolter, D. J., & Hanson, N. D. (2009). Antibacterial-Resistant *Pseudomonas aeruginosa*: Clinical Impact and Complex Regulation of Chromosomally Encoded Resistance Mechanisms. *Clin Microbiol Rev, 22*(4), 582-610. doi: 10.1128/cmr.00040-09.

Livermore, D. (2008). Defining an extended-spectrum β-lactamase. *Clinical Microbiology and Infection, 14*, 3-10.

Livermore, D. M. (1995). beta-Lactamases in laboratory and clinical resistance. *Clin Microbiol Rev, 8*(4), 557-584.

Livermore, D. M. (2001a). Facing antibiotic resistance. *West Indian Med J, 50*(1), 5-7.

Livermore, D. M. (2001b). Of Pseudomonas, porins, pumps and carbapenems. *J Antimicrob Chemother, 47*(3), 247-250.

Livermore, D. M. (2002). Multiple mechanisms of antimicrobial resistance in *Pseudomonas aeruginosa*: our worst nightmare? *Clin Infect Dis, 34*(5), 634-640. doi: CID011143 [pii]10.1086/338782.

Livermore, D. M. (2012). Fourteen years in resistance. *Int J Antimicrob Agents*.

Livermore, D. M., & Brown, D. F. (2001). Detection of beta-lactamase-mediated resistance. *J Antimicrob Chemother, 48 Suppl 1*, 59-64.

Livermore, D. M., Canton, R., Gniadkowski, M., Nordmann, P., Rossolini, G. M., Arlet, G.*, et al.* (2007). CTX-M: changing the face of ESBLs in Europe. *J Antimicrob Chemother, 59*(2), 165-174.

Livermore, D. M., Warner, M., Hall, L. M., Enne, V. I., Projan, S. J., Dunman, P. M.*, et al.* (2001). Antibiotic resistance in bacteria from magpies (Pica pica) and rabbits (Oryctolagus cuniculus) from west Wales. *Environ Microbiol, 3*(10), 658-661. doi: 239 [pii].

Livermore, D. M., & Yang, Y. J. (1987). β-Lactamase lability and inducer power of newer β-lactam antibiotics in relation to their activity against β-lactamase-inducibility mutants of *Pseudomonas aeruginosa*. *J Infect Dis, 155*(4), 775-782.

Llanes, C., Hocquet, D., Vogne, C., Benali-Baitich, D., Neuwirth, C., & Plésiat, P. (2004). Clinical Strains of *Pseudomonas aeruginosa* Overproducing MexAB-OprM and MexXY Efflux Pumps Simultaneously. *Antimicrobial Agents and Chemotherapy, 48*(5), 1797-1802. doi: 10.1128/aac.48.5.1797-1802.2004.

Llano-Sotelo, B., Azucena, E. F., Jr., Kotra, L. P., Mobashery, S., & Chow, C. S. (2002). Aminoglycosides modified by resistance enzymes display diminished binding to the bacterial ribosomal aminoacyl-tRNA site. *Chem Biol, 9*(4), 455-463. doi: S1074552102001254 [pii].

Lodise Jr, T. P., Lomaestro, B., & Drusano, G. L. (2007). Piperacillin-tazobactam for *Pseudomonas aeruginosa* infection: clinical implications of an extended-infusion dosing strategy. *Clin Infect Dis, 44*(3), 357-363.

Lolans, K., Villegas, M. V., & Quinn, J. P. (2008). *Pseudomonas aeruginosa*: An Understanding of Resistance Issues. *Infect Dis Ther, 48*, 149.

Ma, L., Ishii, Y., Ishiguro, M., Matsuzawa, H., & Yamaguchi, K. (1998). Cloning and sequencing of the gene encoding Toho-2, a class A β-lactamase preferentially inhibited by tazobactam. *Antimicrob Agents Chemother, 42*(5), 1181-1186.

Mahenthiralingam, E., Campbell, M. E., & Speert, D. P. (1994). Nonmotility and phagocytic resistance of *Pseudomonas aeruginosa* isolates from chronically colonized patients with cystic fibrosis. *Infect Immun, 62*(2), 596-605.

Mandell, L. A., Wunderink, R. G., Anzueto, A., Bartlett, J. G., Campbell, G. D., Dean, N. C., *et al.* (2007). Infectious Diseases Society of America/American Thoracic Society consensus guidelines on the management of community-acquired pneumonia in adults. *Clin Infect Dis, 44*(Supplement 2), S27.

Mangoni, M. L., Maisetta, G., Di Luca, M., Gaddi, L. M., Esin, S., Florio, W., *et al.* (2008). Comparative analysis of the bactericidal activities of amphibian peptide analogues against multidrug-resistant nosocomial bacterial strains. *Antimicrob Agents Chemother, 52*(1), 85-91. doi: AAC.00796-07 [pii]10.1128/AAC.00796-07.

Manian, F. A., Meyer, L., Jenne, J., Owen, A., & Taff, T. (1996). Loss of antimicrobial susceptibility in aerobic gram-negative bacilli repeatedly isolated from patients in intensive-care units. *Infect Control Hosp Epidemiol* 222-226.

Mao, W., Warren, M. S., Lee, A., Mistry, A., & Lomovskaya, O. (2001). MexXY-OprM efflux pump is required for antagonism of aminoglycosides by divalent cations in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 45*(7), 2001-2007. doi: 10.1128/AAC.45.7.2001-2007.2001.

Marcel, J., Alfa, M., Baquero, F., Etienne, J., Goossens, H., Harbarth, S., *et al.* (2008). Healthcare-associated infections: think globally, act locally. *Clin Microbiol Infect, 14*(10), 895-907.

Marchandin, H., Jean-Pierre, H., De Champs, C., Sirot, D., Darbas, H., Perigault, P. F., *et al.* (2000). Production of a TEM-24 plasmid-mediated extended-spectrum beta-lactamase by a clinical isolate of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 44*(1), 213-216.

Marra, A. R., Pereira, C. A. P., Gales, A. C., Menezes, L. C. C., Ruy Guilherme R., de Souza, J. M. A., Edmond, M. B., *et al.* (2006). Bloodstream Infections with Metallo-beta-Lactamase-Producing *Pseudomonas aeruginosa*: Epidemiology, Microbiology, and Clinical Outcomes. *Antimicrob. Agents Chemother., 50*(1), 388-390.

Martin, E., Stryjewski, M. D., Daniel, J., & Sexton, J. (2003). *Pseudomonas Aeruginosa* Infections in Specific Types of Patients and Clinical Settings. In A. R. Hauser & J. Rello (Eds.), (Vol. 7, pp. 1-15): Springer US.

Martin, S. J., & Yost, R. J. (2011). Infectious Diseases in the Critically Ill Patients. *J Pharm Pract, 24*(1), 35-43.

Martínez-Solano, L., Macia, M. D., Fajardo, A., Oliver, A., & Martinez, J. L. (2008). Chronic *Pseudomonas aeruginosa* Infection in Chronic Obstructive Pulmonary Disease. *Clin Infect Dis, 47*(12), 1526-1533.

Maseda, H., Yoneyama, H., & Nakae, T. (2000). Assignment of the substrate-selective subunits of the MexEF-OprN multidrug efflux pump of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 44*(3), 658-664.

Masood, S., Aslam, N. (2010). In Vitro Susceptibility Test of Different Clinical Isolates against Ceftriaxone. *Oman Med J, 25*(3).

Masuda, N., Sakagawa, E., & Ohya, S. (1995). Outer membrane proteins responsible for multiple drug resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 39*(3), 645-649.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., & Nishino, T. (2000a). Contribution of the MexX-MexY-oprM efflux system to intrinsic resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 44*(9), 2242-2246.

Masuda, N., Sakagawa, E., Ohya, S., Gotoh, N., Tsujimoto, H., & Nishino, T. (2000b). Substrate specificities of MexAB-OprM, MexCD-OprJ, and MexXY-oprM efflux pumps in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 44*(12), 3322-3327.

Matthaiou, D. K., Michalopoulos, A., Rafailidis, P. I., Karageorgopoulos, D. E., Papaioannou, V., Ntani, G.*, et al.* (2008). Risk factors associated with the isolation of colistin-resistant gram-negative bacteria: a matched case-control study. *Crit Care Med, 36*(3), 807.

Medeiros, A. A. (1984). β-Lactamases. *British medical bulletin, 40*(1), 18-27.

Merz, A. J., So, M., & Sheetz, M. P. (2000). Pilus retraction powers bacterial twitching motility. [10.1038/35024105]. *Nature, 407*(6800), 98-102.

Mesaros, N., Nordmann, P., Plesiat, P., Roussel-Delvallez, M., Van Eldere, J., Glupczynski, Y.*, et al.* (2007). *Pseudomonas aeruginosa*: resistance and therapeutic options at the turn of the new millennium. *Clin Microbiol Infect, 13*(6), 560-578.

Micek, S. T., Lloyd, A. E., Ritchie, D. J., Reichley, R. M., Fraser, V. J., & Kollef, M. H. (2005). *Pseudomonas aeruginosa* bloodstream infection: importance of appropriate initial antimicrobial treatment. *Antimicrob Agents Chemother, 49*(4), 1306-1311.

Michalopoulos, A., & Falagas, M. E. (2008). Colistin and polymyxin B in critical care. *Crit Care Clin, 24*(2), 377-391, x.

Mine, T., Morita, Y., Kataoka, A., Mizushima, T., & Tsuchiya, T. (1999). Expression in Escherichia coli of a new multidrug efflux pump, MexXY, from *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 43*(2), 415-417.

Mirsalehian, A., Feizabadi, M., Nakhjavani, F. A., Jabalameli, F., Goli, H., & Kalantari, N. (2010). Detection of VEB-1, OXA-10 and PER-1 genotypes in extended-spectrum β-lactamase-producing-*Pseudomonas aeruginosa* strains isolated from burn patients. *Burns, 36*(1), 70-74.

Mittal, R., Aggarwal, S., Sharma, S., Chhibber, S., & Harjai, K. (2009). Urinary tract infections caused by *Pseudomonas aeruginosa*: A minireview. *2*(3), 101-111.

Mokhonov, V. V., Mokhonova, E. I., Akama, H., & Nakae, T. (2004). Role of the membrane fusion protein in the assembly of resistance-nodulation-cell division multidrug efflux pump in *Pseudomonas aeruginosa*. *Biochem Biophys Res Commun, 322*(2), 483-489.

Monnet, D. L., Biddle, J. W., Edwards, J. R., Culver, D. H., Tolson, J. S., Martone, W. J.*, et al.* (1997). Evidence of interhospital transmission of extended-spectrum beta-lactam-resistant *Klebsiella pneumoniae* in the United States, 1986 to 1993. The National Nosocomial Infections Surveillance System. *Infect Control Hosp Epidemiol, 18*(7), 492-498.

Monstein, H. J., Ostholm-Balkhed, A., Nilsson, M. V., Nilsson, M., Dornbusch, K., & Nilsson, L. E. (2007). Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in *Enterobacteriaceae*. *APMIS, 115*(12), 1400-1408.

Moore, N. M., & Flaws, M. L. (2011a). Antimicrobial Resistance Mechanisms in *Pseudomonas aeruginosa*. *Clin Lab Sci,24*(1), 47.

Moore, N. M., & Flaws, M. L. (2011b). Epidemiology and pathogenesis of *Pseudomonas aeruginosa* infections. *Clin Lab Sci, 24*(1), 43.

Moore, N. M., & Flaws, M. L. (2011c). Treatment Strategies and Recommendations for *Pseudomonas aeruginosa* Infections. *Clin Lab Sci, 24*(1), 52.

Muir, A., & Weinbren, M. J. (2010). New Delhi metallo-beta-lactamase: a cautionary tale. *J Hosp Infect, 75*(3), 239-240.

Mushtaq, S., Ge, Y., & Livermore, D. M. (2004). Doripenem versus *Pseudomonas aeruginosa* in vitro: activity against characterized isolates, mutants, and transconjugants and resistance selection potential. *Antimicrob Agents Chemother, 48*(8), 3086-3092.

Naas, T., Benaoudia, F., Massuard, S., & Nordmann, P. (2000). Integron-located VEB-1 extended-spectrum beta-lactamase gene in a Proteus mirabilis clinical isolate from Vietnam. *J Antimicrob Chemother, 46*(5), 703-711.

Naas, T., & Nordmann, P. (1999). OXA-Type p-Lactamases. *Curr Pharm Des 5*(11), 865-879.

Naas, T., Poirel, L., Karim, A., & Nordmann, P. (1999). Molecular characterization of In50, a class 1 integron encoding the gene for the extended-spectrum β-lactamase VEB-1 in *Pseudomonas aeruginosa*. *FEMS Microbiol Lett, 176*(2), 411-419.

Nadeem Sajjad Raja, N. N. S. (2007). Antimicrobial susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital. *J Microbiol Immunol Infect 40*(1), 45-49.

Nakajima, A., Sugimoto, Y., Yoneyama, H., & Nakae, T. (2002). High-level fluoroquinolone resistance in *Pseudomonas aeruginosa* due to interplay of

the MexAB-OprM efflux pump and the DNA gyrase mutation. *Microbiol Immunol, 46*(6), 391-395.

Nasrin, T., Alam Jilani, M. S., Barai, L., & Haq, J. A. (2011). *Metallo-ß-Lactamase Producing Pseudomonas species in a Tertiary Care Hospital of Dhaka City* (Vol. *4*).

Navaneeth, B., Sridaran, D., Sahay, D., & Belwadi, M. (2002). A preliminary study on metallo-beta-lactamase producing *Pseudomonas aeruginosa* in hospitalized patients. *Indian J Med Res, 116*, 264.

Neonakis, I. K., Scoulica, E. V., Dimitriou, S. K., Gikas, A. I., & Tselentis, Y. J. (2003). Molecular epidemiology of extended-spectrum beta-lactamases produced by clinical isolates in a university hospital in Greece: detection of SHV-5 in *Pseudomonas aeruginosa* and prevalence of SHV-12. *Microb Drug Resist, 9*(2), 161-165.

Neuhauser, M. M., Weinstein, R. A., Rydman, R., Danziger, L. H., Karam, G., & Quinn, J. P. (2003). Antibiotic resistance among gram-negative bacilli in US intensive care units. *JAMA, 289*(7), 885-888.

Neuwirth, C., Siebor, E., Duez, J. M., Pechinot, A., & Kazmierczak, A. (1995). Imipenem resistance in clinical isolates of *Proteus mirabilis* associated with alterations in penicillin-binding proteins. *J Antimicrob Chemother, 36*(2), 335-342.

Nicolas-Chanoine, M. H., & Jarlier, V. (2008). Extended-spectrum β-lactamases in long-term-care facilities. *Clinical Microbiology and Infection, 14*, 111-116.

Niederman, M. S. (2010). Hospital-Acquired Pneumonia, Health Care-Associated Pneumonia, Ventilator-Associated Pneumonia, and Ventilator-Associated Tracheobronchitis: Definitions and Challenges in Trial Design. *Clin Microbiol Infect, 51*(Supplement 1), S12-S17. doi: 10.1086/653035.

Nikaido, H. (1989). Outer membrane barrier as a mechanism of antimicrobial resistance. *Antimicrob Agents Chemother, 33*(11), 1831-1836.

Nordmann, P., & Guibert, M. (1998). Extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*. *J Antimicrob Chemother, 42*(2), 128-131.

Nordmann, P., & Naas, T. (1994). Sequence analysis of PER-1 extended-spectrum beta-lactamase from *Pseudomonas aeruginosa* and comparison with class A beta-lactamases. *Antimicrob Agents Chemother, 38*(1), 104-114.

Nordmann, P., & Poirel, L. (2002). Emerging carbapenemases in Gram-negative aerobes. *Clin Microbiol Infect, 8*(6), 321-331. doi: 401 [pii].

Obritsch, M. D., Fish, D. N., MacLaren, R., & Jung, R. (2004). National surveillance of antimicrobial resistance in *Pseudomonas aeruginosa* isolates obtained from intensive care unit patients from 1993 to 2002. *Antimicrob Agents Chemother, 48*(12), 4606-4610.

Obritsch, M. D., Fish, D. N., MacLaren, R., & Jung, R. (2005). Nosocomial Infections Due to Multidrug-Resistant *Pseudomonas aeruginosa*: Epidemiology and Treatment Options. *Pharmacotherapy, 25*(10), 1353-1364.

Ochs, M. M., Bains, M., & Hancock, R. E. (2000). Role of putative loops 2 and 3 in imipenem passage through the specific porin OprD of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 44*(7), 1983-1985.

Ochs, M. M., McCusker, M. P., Bains, M., & Hancock, R. E. (1999). Negative regulation of the *Pseudomonas aeruginosa* outer membrane porin OprD selective for imipenem and basic amino acids. *Antimicrob Agents Chemother, 43*(5), 1085-1090.

Okamoto, K., Gotoh, N., & Nishino, T. (2001). *Pseudomonas aeruginosa* reveals high intrinsic resistance to penem antibiotics: penem resistance mechanisms and their interplay. *Antimicrob Agents Chemother, 45*(7), 1964-1971. doi: 10.1128/AAC.45.7.1964-1971.2001.

Okamoto, K., Gotoh, N., & Nishino, T. (2002). Extrusion of penem antibiotics by multicomponent efflux systems MexAB-OprM, MexCD-OprJ, and MexXY-OprM of *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 46*(8), 2696-2699.

Olayinka, A., Onile, B., & Olayinka, B. (2004). Prevalence of multi-drug resistant (mdr) *pseudomonas aeruginosa* isolates in surgical units of ahmadu bello university teaching hospital, zaria, nigeria: an indication for effective control measures. *Ann Afr Med, 3*, 1.

Ozer, B., Duran, N., Onlen, Y., & Savas, L. (2012). Efflux pump genes and antimicrobial resistance of *Pseudomonas aeruginosa* strains isolated from lower respiratory tract infections acquired in an intensive care unit. *J Antibiot (Tokyo), 65*(1), 9-13.

Ozgumus, O. B., Caylan, R., Tosun, I., Sandalli, C., Aydin, K., & Koksal, I. (2007). Molecular epidemiology of clinical *Pseudomonas aeruginosa* isolates carrying IMP-1 metallo-beta-lactamase gene in a University Hospital in Turkey. *Microb Drug Resist, 13*(3), 191-198. doi: 10.1089/mdr.2007.748.

Pagani, L., Colinon, C., Migliavacca, R., Labonia, M., Docquier, J. D., Nucleo, E.*, et al.* (2005). Nosocomial outbreak caused by multidrug-resistant *Pseudomonas aeruginosa* producing IMP-13 metallo-beta-lactamase. *J Clin Microbiol, 43*(8), 3824-3828. doi: 43/8/3824 [pii]10.1128/JCM.43.8.3824-3828.2005.

Pagani, L., Mantengoli, E., Migliavacca, R., Nucleo, E., Pollini, S., Spalla, M.*, et al.* (2004). Multifocal detection of multidrug-resistant *Pseudomonas aeruginosa* producing the PER-1 extended-spectrum beta-lactamase in Northern Italy. *J Clin Microbiol, 42*(6), 2523-2529.

Pai, H., Kim, J., Lee, J. H., Choe, K. W., & Gotoh, N. (2001). Carbapenem resistance mechanisms in *Pseudomonas aeruginosa* clinical isolates.

Antimicrob Agents Chemother, 45(2), 480-484. doi: 10.1128/AAC.45.2.480-484.2001.

Palleroni, N. J. (1984). Genus I. *Pseudomonas* Migula 1894. In: *N. R. Kreig and J. G. Holt (Eds)Bergey's Manual of Systematic Bacteriology* (pp. 141–199): Williams and Wilkins. Baltimore, MA.

Paramythiotou, E., Lucet, J. C., Timsit, J. F., Vanjak, D., Paugam-Burtz, C., Trouillet, J. L.*, et al.* (2004). Acquisition of multidrug-resistant *Pseudomonas aeruginosa* in patients in intensive care units: role of antibiotics with antipseudomonal activity. *Clin Infect Dis, 38*(5), 670.

Park, Y. S., Lee, H., Chin, B. S., Han, S. H., Hong, S. G., Hong, S. K.*, et al.* (2011). Acquisition of extensive drug-resistant *Pseudomonas aeruginosa* among hospitalized patients: risk factors and resistance mechanisms to carbapenems. *J Hosp Infect, 79*(1), 54-58. doi: 10.1016/j.jhin.2011.05.014.

Paterson, D. L., & Bonomo, R. A. (2005). Extended-spectrum β-lactamases: a clinical update. *Clin Microbiol Rev, 18*(4), 657-686.

Paterson, D. L., Hujer, K. M., Hujer, A. M., Yeiser, B., Bonomo, M. D., Rice, L. B.*, et al.* (2003). Extended-spectrum beta-lactamases in *Klebsiella pneumoniae* bloodstream isolates from seven countries: dominance and widespread prevalence of SHV- and CTX-M-type beta-lactamases. *Antimicrob Agents Chemother, 47*(11), 3554-3560.

Pathmanathan, S., Samat, N., & Mohamed, R. (2009). Antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* from a Malaysian Hospital. *Malays J Med Sci, 16*(2).

Paul, M., Benuri-Silbiger, I., Soares-Weiser, K., & Leibovici, L. (2004). β lactam monotherapy versus β lactam-aminoglycoside combination therapy for sepsis in immunocompetent patients: systematic review and meta-analysis of randomised trials. *BMJ, 328*(7441), 668.

Peña, C., Pujol, M., Ardanuy, C., Ricart, A., Pallares, R., Liñares, J.*, et al.* (1998). Epidemiology and successful control of a large outbreak due to *Klebsiella pneumoniae* producing extendedspectrum β-lactamases. *Antimicrob Agents Chemother, 42*(1), 53-58.

Pfaffl, M. W., Horgan, G. W., & Dempfle, L. (2002). Relative expression software tool (REST©) for group-wise comparison and statistical analysis of relative expression results in real-time PCR. Nucleic Acids Res*, 30*(9), e36-e36.

Pfaller, M. A., & Segreti, J. (2006). Overview of the Epidemiological Profileand Laboratory Detection of Extended-Spectrum β-Lactamases. *Clin Infect Dis, 42*(Supplement 4), S153.

Pfeifer, Y., Cullik, A., & Witte, W. (2010). Resistance to cephalosporins and carbapenems in Gram-negative bacterial pathogens. *Int J Med Microbiol, 300*(6), 371-379.

Philippon, A., Arlet, G., & Jacoby, G. A. (2002). Plasmid-determined AmpC-type β-lactamases. *Antimicrob Agents Chemother, 46*(1), 1-11.

Piddock, L. J. V. (2011). The crisis of no new antibiotics—what is the way forward? *Lancet Infect Dis*.

Pirnay, J.-P., De Vos, D., Cochez, C., Bilocq, F., Pirson, J., Struelens, M.*, et al.* (2003). Molecular Epidemiology of *Pseudomonas aeruginosa* Colonization in a Burn Unit: Persistence of a Multidrug-Resistant Clone and a Silver Sulfadiazine-Resistant Clone. *J. Clin. Microbiol., 41*(3), 1192-1202. doi: 10.1128/jcm.41.3.1192-1202.2003.

Pirnay, J. P., De Vos, D., Mossialos, D., Vanderkelen, A., Cornelis, P., & Zizi, M. (2002). Analysis of the *Pseudomonas aeruginosa* OprD gene from clinical and environmental isolates. *Environ Microbiol, 4*(12), 872-882. doi: 281 [pii].

Pitout, J. D. D., Gregson, D. B., Poirel, L., McClure, J.-A., Le, P., & Church, D. L. (2005). Detection of *Pseudomonas aeruginosa* Producing Metallo-β-Lactamases in a Large Centralized Laboratory. *J Med Microbio, 43*(7), 3129-3135. doi: 10.1128/jcm.43.7.3129-3135.2005.

Podbielski, A., Schonling, J., Melzer, B., Warnatz, K., & Leusch, H. G. (1991). Molecular characterization of a new plasmid-encoded SHV-type beta-lactamase (SHV-2 variant) conferring high-level cefotaxime resistance upon *Klebsiella pneumoniae*. *J Gen Microbiol, 137*(3), 569-578.

Poirel, L., Lebessi, E., Castro, M., Fevre, C., Foustoukou, M., & Nordmann, P. (2004a). Nosocomial outbreak of extended-spectrum beta-lactamase SHV-5-producing isolates of *Pseudomonas aeruginosa* in Athens, Greece. *Antimicrob Agents Chemother, 48*(6), 2277-2279. doi: 10.1128/AAC.48.6.2277-2279.200448/6/2277 [pii].

Poirel, L., Magalhaes, M., Lopes, M., & Nordmann, P. (2004b). Molecular analysis of metallo-beta-lactamase gene bla(SPM-1)-surrounding sequences from disseminated *Pseudomonas aeruginos* isolates in Recife, Brazil. *Antimicrob Agents Chemother, 48*(4), 1406-1409.

Poirel, L., & Nordmann, P. (2002). Acquired carbapenem-hydrolyzing beta-lactamases and their genetic support. *Curr Pharm Biotechnol, 3*(2), 117-127.

Poirel, L., Pitout, J. D., & Nordmann, P. (2007). Carbapenemases: molecular diversity and clinical consequences. *Future Microbiol, 2*(5), 501-512.

Poirel, L., Walsh, T. R., Cuvillier, V., & Nordmann, P. (2011). Multiplex PCR for detection of acquired carbapenemase genes. *Diagn Microbiol Infect Dis, 70*(1), 119-123. doi: 10.1016/j.diagmicrobio.2010.12.002.

Poole, K. (2002). Outer membranes and efflux: the path to multidrug resistance in Gram-negative bacteria. *Curr Pharm Biotechnol, 3*(2), 77-98.

Poole, K. (2004a). Efflux-mediated multiresistance in Gram-negative bacteria. *Clin Microbiol Infect, 10*(1), 12-26. doi: 763 [pii].

Poole, K. (2004b). Resistance to beta-lactam antibiotics. *Cell Mol Life Sci, 61*, 2200 - 2223.

Poole, K. (2007). Efflux pumps as antimicrobial resistance mechanisms. *Ann Med, 39*(3), 162-176. doi: 777631811 [pii]10.1080/07853890701195262.

Poole, K. (2011). *Pseudomonas aeruginosa*: resistance to the max. [Review]. *Front Microbiol, 2*. doi: 10.3389/fmicb.2011.00065.

Poole, K., Gotoh, N., Tsujimoto, H., Zhao, Q., Wada, A., Yamasaki, T.*, et al.* (1996a). Overexpression of the mexC-mexD-oprJ efflux operon in nfxB-type multidrug-resistant strains of *Pseudomonas aeruginosa*. *Mol Microbiol, 21*(4), 713-724.

Poole, K., & Srikumar, R. (2001). Multidrug efflux in Pseudomonas aeruginosa: components, mechanisms and clinical significance. *Curr Top Med Chem, 1*, 59 - 71.

Poole, K., Tetro, K., Zhao, Q., Neshat, S., Heinrichs, D. E., & Bianco, N. (1996b). Expression of the multidrug resistance operon mexA-mexB-oprM in *Pseudomonas aeruginosa*: mexR encodes a regulator of operon expression. *Antimicrob Agents Chemother, 40*(9), 2021-2028.

Quale, J., Bratu, S., Gupta, J., & Landman, D. (2006). Interplay of Efflux System, ampC, and oprD Expression in Carbapenem Resistance of *Pseudomonas aeruginosa* Clinical Isolates. *Antimicrob. Agents Chemother., 50*(5), 1633-1641. doi: 10.1128/aac.50.5.1633-1641.2006.

Queenan, A. M., Shang, W., Bush, K., & Flamm, R. K. (2010). Differential selection of single-step AmpC or efflux mutants of *Pseudomonas aeruginosa* by using cefepime, ceftazidime, or ceftobiprole. *Antimicrob. Agents Chemother, 54*(10), 4092-4097.

Quinn, J. P., Dudek, E. J., DiVincenzo, C. A., Lucks, D. A., & Lerner, S. A. (1986). Emergence of resistance to imipenem during therapy for *Pseudomonas aeruginosa* infections. *J Infect Dis, 154*(2), 289-294.

Raja, N., & Singh, N. (2007). Antimicrobial susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital. *J Microbiol Immunol Infect, 40*(1), 45.

Rice, L. (2002). Association of different mobile elements to generate novel integrative elements. *Cell Mol Life Sci, 59*(12), 2023-2032.

Rice, L. B. (2006). Challenges in identifying new antimicrobial agents effective for treating infections with Acinetobacter baumannii and *Pseudomonas aeruginosa*. *Clin Infect Dis, 43*(Supplement 2), S100.

Rice, L. B. (2008). Federal funding for the study of antimicrobial resistance in nosocomial pathogens: no ESKAPE. *J Infect Dis, 197*(8), 1079-1081.

Rice, L. B., Eckstein, E. C., DeVente, J., & Shlaes, D. M. (1996). Ceftazidime-resistant *Klebsiella pneumoniae* isolates recovered at the Cleveland Department of Veterans Affairs Medical Center. *Clin Infect Dis, 23*(1), 118-124.

Rios, F., Luna, C., Maskin, B., Valiente, A. S., Lloria, M., Gando, S.*, et al.* (2007). Ventilator-associated pneumonia due to colistin susceptible-only microorganisms. *Eur Respir J, 30*(2), 307-313.

Rodríguez-Baño, J., Navarro, M. D., Romero, L., Martínez-Martínez, L., Muniain, M. A., Perea, E. J.*, et al.* (2004). Epidemiology and clinical features of infections caused by extended-spectrum beta-lactamase-producing *Escherichia coli* in nonhospitalized patients. *J Med Microbio, 42*(3), 1089-1094.

Rodríguez-Baño, J., Navarro, M. D., Romero, L., Muniain, M. A., Perea, E. J., Pérez-Cano, R.*, et al.* (2006). Clinical and molecular epidemiology of extended-spectrum β-lactamase producing *Escherichia coli* as a cause of nosocomial infection or colonization: implications for control. *Clin Infect Dis, 42*(1), 37.

Rodríguez-Martínez, J.-M., Poirel, L., & Nordmann, P. (2009). Extended-Spectrum Cephalosporinases in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 53*(5), 1766-1771. doi: 10.1128/aac.01410-08

Rodriguez-Martinez, J., Poirel, L., & Nordmann, P. (2009). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 53*, 4783 - 4788.

Rolain, J. M., Parola, P., & Cornaglia, G. (2010). New Delhi metallo-beta-lactamase (NDM-1): towards a new pandemia? *Clin Microbiol Infect, 16*(12), 1699-1701. doi: 10.1111/j.1469-0691.2010.03385.x.

Rossolini, G., & Mantengoli, E. (2005). Treatment and control of severe infections caused by multiresistant *Pseudomonas aeruginosa*. *Clin Microbiol Infect, 11*, 17-32.

Rowe-Magnus, D. A., & Mazel, D. (2002). The role of integrons in antibiotic resistance gene capture. *Int J Med Microbiol, 292*(2), 115-125.

Roy-Burman, A., Savel, R. H., Racine, S., Swanson, B. L., Revadigar, N. S., Fujimoto, J.*, et al.* (2001). Type III protein secretion is associated with death in lower respiratory and systemic *Pseudomonas aeruginosa* infections. *J Infect Dis, 183*(12), 1767-1774.

Ruiz, M., Ewig, S., Torres, A., Arancibia, F., Marco, F., Mensa, J.*, et al.* (1999). Severe community-acquired pneumonia. *Am J Respir Crit Care Med, 160*(3), 923-929.

Sadikot, R. T., Blackwell, T. S., Christman, J. W., & Prince, A. S. (2005). Pathogen–host interactions in *Pseudomonas aeruginosa* pneumonia. *Am J Respir Crit Care Med, 171*(11), 1209-1223.

Safdar, N., Handelsman, J., & Maki, D. G. (2004). Does combination antimicrobial therapy reduce mortality in Gram-negative bacteraemia? A meta-analysis. *Lancet Infect Dis, 4*(8), 519-527.

Sanchez, L., Pan, W., Vinas, M., & Nikaido, H. (1997). The acrAB homolog of *Haemophilus influenzae* codes for a functional multidrug efflux pump. *J Bacteriol, 179*(21), 6855-6857.

Santos Filho, L., Eagye, K., Kuti, J., & Nicolau, D. (2007). Addressing resistance evolution in *Pseudomonas aeruginosa* using pharmacodynamic modelling: application to meropenem dosage and combination therapy. *Clin Microbiol Infect, 13*(6), 579-585.

Savli, H., Karadenizli, A., Kolayli, F., Gundes, S., Ozbek, U., & Vahaboglu, H. (2003). Expression stability of six housekeeping genes: a proposal for resistance gene quantification studies of *Pseudomonas aeruginosa* by real-time quantitative RT-PCR. *J Med Microbiol, 52*(5), 403-408. doi: 10.1099/jmm.0.05132-0.

Scarff, J., & Goldberg, J. (2008). Vaccination against *Pseudomonas aeruginosa* pneumonia in immunocompromised mice. *Clin Vaccine Immunol, 15*(2), 367.

Scheetz, M. H., Hoffman, M., Bolon, M. K., Schulert, G., Estrellado, W., Baraboutis, I. G.*, et al.* (2009). Morbidity associated with *Pseudomonas aeruginosa* bloodstream infections. *Diagn Microbiol Infect Dis, 64*(3), 311-319.

Schulert, G. S., Feltman, H., Rabin, S. D. P., Martin, C. G., Battle, S. E., Rello, J.*, et al.* (2003). Secretion of the toxin ExoU is a marker for highly virulent *Pseudomonas aeruginosa* isolates obtained from patients with hospital-acquired pneumonia. *J Infect Dis, 188*(11), 1695-1706.

Schweizer, H. P. (2003). Efflux as a mechanism of resistance to antimicrobials in *Pseudomonas aeruginosa* and related bacteria: unanswered questions. *Genet Mol Res, 2*(1), 48-62. doi: S01 [pii].

Shahid, M., Sobia, F., Singh, A., Malik, A., Khan, H., Jonas, D.*, et al.* (2009). Beta-lactams and beta-lactamase-inhibitors in current-or potential-clinical practice: a comprehensive update. *Crit Rev Microbiol, 35*(2), 81-108.

Shannon, K. P., King, A., Phillips, I., Nicolas, M. H., & Philippon, A. (1990). Importance of organisms producing broad-spectrum SHV-group beta-lactamases into the United Kingdom. *J Antimicrob Chemother, 25*(3), 343-351.

Shibata, N., Doi, Y., Yamane, K., Yagi, T., Kurokawa, H., Shibayama, K.*, et al.* (2003). PCR typing of genetic determinants for metallo-beta-lactamases and

integrases carried by gram-negative bacteria isolated in Japan, with focus on the class 3 integron. *J Clin Microbiol, 41*(12), 5407-5413.

Siegel, J. D., Rhinehart, E., Jackson, M., Chiarello, L., & Committee, H. I. C. P. A. (2007). Management of multidrug-resistant organisms in health care settings, 2006. *Am J Infect Control, 35*(2), 165.

Siegman-Igra, Y., Ravona, R., Primerman, H., & Giladi, M. (1998). *Pseudomonas aeruginosa* bacteremia: an analysis of 123 episodes, with particular emphasis on the effect of antibiotic therapy. *Int J Infect Dis 2*(4), 211-215.

Sirot, D., Sirot, J., Labia, R., Morand, A., Courvalin, P., Darfeuille-Michaud, A.*, et al.* (1987a). Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel beta-lactamase. *J Antimicrob Chemother, 20*(3), 323-334.

Sirot, D., Sirot, J., Labia, R., Morand, A., Courvalin, P., Darfeuille-Michaud, A.*, et al.* (1987b). Transferable resistance to third-generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. *J Antimicrob Chemother, 20*(3), 323-334.

Sivanmaliappan, T. S., & Sevanan, M. (2011). Antimicrobial Susceptibility Patterns of *Pseudomonas aeruginosa* from Diabetes Patients with Foot Ulcers. *Int J Microbiol, 2011*, 605195. doi: 10.1155/2011/605195.

Sobel, M. L., McKay, G. A., & Poole, K. (2003). Contribution of the MexXY multidrug transporter to aminoglycoside resistance in *Pseudomonas aeruginosa* clinical isolates. *Antimicrob Agents Chemother, 47*(10), 3202-3207.

Sobieszczyk, M. E., Furuya, E. Y., Hay, C. M., Pancholi, P., Della-Latta, P., Hammer, S. M.*, et al.* (2004). Combination therapy with polymyxin B for the treatment of multidrug-resistant Gram-negative respiratory tract infections. *Journal of Antimicrobial Chemotherapy, 54*(2), 566-569. doi: 10.1093/jac/dkh369.

Sougakoff, W., Goussard, S., & Courvalin, P. (1988a). The TEM-3 [beta]-lactamase, which hydrolyzes broad-spectrum cephalosporins, is derived from the TEM-2 penicillinase by two amino acid substitutions. *FEMS Microbiol Lett, 56*(3), 343-348.

Sougakoff, W., Goussard, S., Gerbaud, G., & Courvalin, P. (1988b). Plasmid-mediated resistance to third-generation cephalosporins caused by point mutations in TEM-type penicillinase genes. *Rev Infect Dis, 10*(4), 879-884.

Speert, D. P., Campbell, M. E., Henry, D. A., Milner, R., Taha, F., Gravelle, A.*, et al.* (2002). Epidemiology of *Pseudomonas aeruginosa* in Cystic Fibrosis in British Columbia, Canada. *Am. J. Respir. Crit. Care Med., 166*(7), 988-993. doi: 10.1164/rccm.2203011.

Spellberg, B., Blaser, M., Guidos, R., Boucher, H., Bradley, J., Eisenstein, B., *et al.* (2011). Combating antimicrobial resistance: policy recommendations to save lives. *Clin Infect Dis, 52*(Suppl 5), S397-428.

Spratt, B. G. (1977). Properties of the penicillin-binding proteins of *Escherichia coli* K12. *Eur J Biochem, 72*(2), 341-352.

Srikumar, R., Li, X. Z., & Poole, K. (1997). Inner membrane efflux components are responsible for beta-lactam specificity of multidrug efflux pumps in *Pseudomonas aeruginosa*. *J Bacteriol, 179*(24), 7875-7881.

Stein, G. E. (2005). Antimicrobial resistance in the hospital setting: impact, trends, and infection control measures. *Pharmacotherapy, 25*(10P2), 44S-54S.

Strateva, T., Ouzounova-Raykova, V., Markova, B., Todorova, A., Marteva-Proevska, Y., & Mitov, I. (2007). Widespread detection of VEB-1-type extended-spectrum beta-lactamases among nosocomial ceftazidime-resistant *Pseudomonas aeruginosa* isolates in Sofia, Bulgaria. *J Chemother, 19*(2), 140-145.

Strateva, T., & Yordanov, D. (2009). *Pseudomonas aeruginosa* - a phenomenon of bacterial resistance. *J Med Microbiol, 58*(Pt 9), 1133-1148. doi: jmm.0.009142-0 [pii]10.1099/jmm.0.009142-0.

Surveillance, N. N. I. (2004). System Report, data summary from January 1992 through June 2004, issued October 2004. *Am J Infect Control, 32*(8).

System, A. r. f. t. N. (2003). National Nosocomial Infections Surveillance (NNIS) System Report, data summary from January 1992 through June 2003, issued August 2003. *Am J Infect Control, 31*(8), 481-498.

Talon, d., Mulin, b., Rouget, c., Bailly, p., Thouverez, m., & Viel, j.-f. (1998). Risks and Routes for Ventilator-Associated Pneumonia with *Pseudomonas aeruginosa*. *Am. J. Respir. Crit. Care Med., 157*(3), 978-984.

Tam, V., Schilling, A., LaRocco, M., Gentry, L., Lolans, K., Quinn, J., *et al.* (2007). Prevalence of AmpC over-expression in bloodstream isolates of *Pseudomonas aeruginosa*. *Clin Microbiol Infect, 13*(4), 413-418.

Tam, V. H., Gamez, E. A., Weston, J. S., Gerard, L. N., LaRocco, M. T., Caeiro, J. P., *et al.* (2008). Outcomes of bacteremia due to *Pseudomonas aeruginosa* with reduced susceptibility to piperacillin-tazobactam: implications on the appropriateness of the resistance breakpoint. *Clin Infect Dis, 46*(6), 862.

Tam, V. H., Schilling, A. N., Vo, G., Kabbara, S., Kwa, A. L., Wiederhold, N. P., *et al.* (2005). Pharmacodynamics of polymyxin B against *Pseudomonas aeruginosa. Antimicrob Agents Chemother, 49*(9), 3624-3630. doi: 49/9/3624 [pii]10.1128/AAC.49.9.3624-3630.2005.

Tennenberg, A. M., Davis, N. B., Wu, S. C., & Kahn, J. (2006). Pneumonia due to *Pseudomonas aeruginosa*: the levofloxacin clinical trials experience. *Curr Med Res Opin ®, 22*(5), 843-850.

Tenover, F. C. (2006). Mechanisms of antimicrobial resistance in bacteria. *Am J Med, 119*(6), S3-S10.

Tenover, F. C., Raney, P. M., Williams, P. P., Rasheed, J. K., Biddle, J. W., Oliver, A.*, et al.* (2003). Evaluation of the NCCLS extended-spectrum beta-lactamase confirmation methods for *Escherichia coli* with isolates collected during Project ICARE. *J Clin Microbiol, 41*(7), 3142-3146.

Tholen, D. (2006). CLSI evaluation protocols. *MLO Med Lab Obs, 38*(8), 38, 40-31.

Thuong, M., Arvaniti, K., Ruimy, R., De la Salmoniere, P., Scanvic-Hameg, A., Lucet, J.*, et al.* (2003). Epidemiology of *Pseudomonas aeruginosa* and risk factors for carriage acquisition in an intensive care unit. *J Hosp Infect, 53*(4), 274-282.

Todar, K. (2006). *Todar's online textbook of bacteriology*: Kenneth Todar, University of Wisconsin-Madison Department of Bacteriology.

Tomas, M., Doumith, M., Warner, M., Turton, J., Beceiro, A., Bou, G.*, et al.* (2010). Efflux pumps, OprD porin, AmpC beta-lactamase, and multiresistance in *Pseudomonas aeruginosa* isolates from cystic fibrosis patients. *Antimicrob Agents Chemother, 54*, 2219 - 2224.

Tzouvelekis, L. S., & Bonomo, R. A. (1999). SHV-type beta-lactamases. *Curr Pharm Des, 5*(11), 847-864.

Ullah, F., Malik, S. A., & Ahmed, J. (2009). Antimicrobial susceptibility and ESBL prevalence in *Pseudomonas aeruginosa* isolated from burn patients in the North West of Pakistan. *Burns, 35*(7), 1020-1025.

Urban, C., Mariano, N., Rahman, N., Queenan, A. M., Montenegro, D., Bush, K.*, et al.* (2000). Detection of multiresistant ceftazidime-susceptible *Klebsiella pneumoniae* isolates lacking TEM-26 after class restriction of cephalosporins. *Microb Drug Resist, 6*(4), 297-303.

Vakulenko, S. B., & Mobashery, S. (2003). Versatility of aminoglycosides and prospects for their future. *Clin Microbiol Rev, 16*(3), 430-450.

Valverde, A., Coque, T. M., García-San Miguel, L., Baquero, F., & Cantón, R. (2008). Complex molecular epidemiology of extended-spectrum β-lactamases in *Klebsiella pneumoniae*: a long-term perspective from a single institution in Madrid. *J Antimicrob Chemother, 61*(1), 64.

Vashist, J., Tiwari, V., Das, R., Kapil, A., & Rajeswari, M. R. (2011). Analysis of penicillin-binding proteins (PBPs) in carbapenem resistant Acinetobacter baumannii. *Indian J Med Res, 133*(3), 332-338. doi: Indian J MedRes_2011_133_3_332_78380 [pii].

Vila, J., & Martinez, J. (2008). Clinical impact of the over-expression of efflux pump in nonfermentative Gram-negative bacilli, development of efflux pump inhibitors. *Curr Drug Targets, 9*, 797 - 807.

Vogne, C., Aires, J. R., Bailly, C., Hocquet, D., & Plesiat, P. (2004). Role of the multidrug efflux system MexXY in the emergence of moderate resistance to aminoglycosides among *Pseudomonas aeruginosa* isolates from patients with cystic fibrosis. *Antimicrob Agents Chemother, 48*(5), 1676-1680.

von Gotz, F., Haussler, S., Jordan, D., Saravanamuthu, S. S., Wehmhoner, D., Strussmann, A.*, et al.* (2004). Expression Analysis of a Highly Adherent and Cytotoxic Small Colony Variant of *Pseudomonas aeruginosa* Isolated from a Lung of a Patient with Cystic Fibrosis. *J. Bacteriol., 186*(12), 3837-3847. doi: 10.1128/jb.186.12.3837-3847.2004.

Walkty, A., DeCorby, M., Nichol, K., Mulvey, M. R., Hoban, D., & Zhanel, G. (2008). Antimicrobial susceptibility of *Pseudomonas aeruginosa* isolates obtained from patients in Canadian intensive care units as part of the Canadian National Intensive Care Unit study. *Diagn Microbiol Infect Dis, 61*(2), 217-221.

Walsh, T. R., Toleman, M. A., Poirel, L., & Nordmann, P. (2005). Metallo-beta-lactamases: the quiet before the storm? *Clin Microbiol Rev, 18*(2), 306-325. doi: 18/2/306 [pii]10.1128/CMR.18.2.306-325.2005.

Wang, J., Zhou, J.-y., Qu, T.-t., Shen, P., Wei, Z.-q., Yu, Y.-s.*, et al.* (2010). Molecular epidemiology and mechanisms of carbapenem resistance in *Pseudomonas aeruginosa* isolates from Chinese hospitals. *Int J Antimicrob Agents, 35*(5), 486-491.

Wang, J. Y., Lee, L. N., Lai, H. C., Wang, S. K., Jan, I. S., Yu, C. J.*, et al.* (2007). Fluoroquinolone resistance in Mycobacterium tuberculosis isolates: associated genetic mutations and relationship to antimicrobial exposure. *J Antimicrob Chemother, 59*(5), 860-865. doi: dkm061 [pii]10.1093/jac/dkm061.

Watanabe, M., Iyobe, S., Inoue, M., & Mitsuhashi, S. (1991). Transferable imipenem resistance in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 35*(1), 147-151.

Weinstein, R. A. (1998). Nosocomial infection update. *Emerging Infect Dis, 4*(3), 416.

Weinstein, R. A., & Hota, B. (2004). Contamination, Disinfection, and Cross-Colonization: Are Hospital Surfaces Reservoirs for Nosocomial Infection? *Clin Infect Dis, 39*(8), 1182-1189. doi: 10.1086/424667.

Weldhagen, G. F., Poirel, L., & Nordmann, P. (2003). Ambler class A extended-spectrum beta-lactamases in *Pseudomonas aeruginosa*: novel developments and clinical impact. *Antimicrob Agents Chemother, 47*(8), 2385-2392.

Wiener, J., Quinn, J. P., Bradford, P. A., Goering, R. V., Nathan, C., Bush, K.*, et al.* (1999). Multiple antibiotic–resistant *Klebsiella* and *Escherichia coli* in nursing homes. *JAMA, 281*(6), 517-523.

Wilson, R., Pitt, T., Taylor, G., Watson, D., MacDermot, J., Sykes, D., *et al.* (1987). Pyocyanin and 1-hydroxyphenazine produced by *Pseudomonas aeruginosa* inhibit the beating of human respiratory cilia in vitro. *J Clin Invest, 79*(1), 221.

Wikler, M. (2008). Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing: Eighteenth Informational Supplement. Wayne: Clinical and Laboratory Standards Institute*.

Wolter, D. J., Smith-Moland, E., Goering, R. V., Hanson, N. D., & Lister, P. D. (2004). Multidrug resistance associated with mexXY expression in clinical isolates of *Pseudomonas aeruginosa* from a Texas hospital. *Diagn Microbiol Infect Dis, 50*(1), 43-50. doi: DOI: 10.1016/j.diagmicrobio.2004.05.004.

Woodford, N., Zhang, J., Kaufmann, M. E., Yarde, S., Tomas, M. M., Faris, C., *et al.* (2008). Detection of *Pseudomonas aeruginosa* isolates producing VEB-type extended-spectrum β-lactamases in the United Kingdom. *J Antimicrob Chemother, 62*(6), 1265.

Wroblewska, M., Rudnicka, J., Marchel, H., & Luczak, M. (2006). Multidrug-resistant bacteria isolated from patients hospitalised in Intensive Care Units. *Int J Antimicrob Agents, 27*(4), 285-289.

Xavier, D., Picao, R., Girardello, R., Fehlberg, L., & Gales, A. (2010). Efflux pumps expression and its association with porin down-regulation and beta-lactamase production among *Pseudomonas aeruginosa* causing bloodstream infections in Brazil. *BMC Microbiol, 10*(1), 217.

Yan, J. J., Ko, W. C., Tsai, S. H., Wu, H. M., & Wu, J. J. (2001). Outbreak of infection with multidrug-resistant *Klebsiella pneumoniae* carrying bla(IMP-8) in a university medical center in Taiwan. *J Clin Microbiol, 39*(12), 4433-4439. doi: 10.1128/JCM.39.12.4433-4439.2001.

Yang, Y., Rasmussen, B. A., & Shlaes, D. M. (1999). Class A β-lactamases enzyme-inhibitor interactions and resistance. *Pharmacol Ther, 83*(2), 141-151. doi: 10.1016/s0163-7258(99)00027-3.

Yatsuyanagi, J., Saito, S., Ito, Y., Ohta, K., Kato, J., Harata, S., *et al.* (2004). Identification of *Pseudomonas aeruginosa* clinical strains harboring the bla(VIM-2) metallo-beta-lactamase gene in Akita Prefecture, Japan. *Jpn J Infect Dis, 57*(3), 130-132.

Yoneda, K., Chikumi, H., Murata, T., Gotoh, N., Yamamoto, H., Fujiwara, H., *et al.* (2005). Measurement of *Pseudomonas aeruginosa* multidrug efflux pumps by quantitative real-time polymerase chain reaction. *FEMS Microbiol Lett, 243*, 125 - 131.

Yoneyama, H., & Nakae, T. (1993). Mechanism of efficient elimination of protein D2 in outer membrane of imipenem-resistant *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother, 37*(11), 2385-2390.

Yong, D., Toleman, M. A., Giske, C. G., Cho, H. S., Sundman, K., Lee, K., *et al.* (2009). Characterization of a new metallo-beta-lactamase gene, bla(NDM-1), and a novel erythromycin esterase gene carried on a unique genetic structure in *Klebsiella pneumoniae* sequence type 14 from India. *Antimicrob Agents Chemother, 53*(12), 5046-5054. doi: AAC.00774-09 [pii]10.1128/AAC.00774-09.

Yoshimura, F., & Nikaido, H. (1985). Diffusion of beta-lactam antibiotics through the porin channels of *Escherichia coli* K-12. *Antimicrob Agents Chemother, 27*(1), 84-92.

Young, E., Mandell, G., Bennett, J., & Dolin, R. (2000). Principles and practice of infectious diseases. *Clin Infect Dis*.

Yuan, M., Aucken, H., Hall, L., Pitt, T., & Livermore, D. (1998). Epidemiological typing of *klebsiellae* with extended-spectrum beta-lactamases from European intensive care units. *J Antimicrob Chemother, 41*(5), 527-539.

Zavascki, A. P., Barth, A. L., Fernandes, J. F., Moro, A. L. D., Gonçalves, A. L. S., & Goldani, L. Z. (2006). Reappraisal of *Pseudomonas aeruginosa* hospital-acquired pneumonia mortality in the era of metallo-β-lactamase-mediated multidrug resistance: a prospective observational study. *Crit Care, 10*(4), R114.

Zuanazzi, D., Souto, R., Mattos, M., Zuanazzi, M., Tura, B., Sansone, C., *et al.* (2010). Prevalence of potential bacterial respiratory pathogens in the oral cavity of hospitalised individuals. *Arch Oral Biol, 55*(1), 21-28.

CHAPTER 5

APPENDICIES

APPENDIX I ETHIC APPROVAL LETTER

UNIVERSITI MALAYA
PUSAT PERUBATAN UM

JAWATANKUASA ETIKA PERUBATAN
PUSAT PERUBATAN UNIVERSITI MALAYA
ALAMAT: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEFON: 03-79493209 FAKSIMILI: 03-79494638

No. Rujukan: PPUM/MDU/300/04/03

21 Februari 2012

Dr. Adbelkodose M Al- Kabsi
Jabatan Mikrobiologi Perubatan
Pusat Perubatan Universiti Malaya

Tuan,

SURAT PEMAKLUMAN KEPUTUSAN PERMOHONAN MENJALANKAN PROJEK PENYELIDIKAN
Multidrug efflux pumps over-expression and porin down- regulation in association with drug resistance among nosocomial pseudomonas aeruginosa isolates in Malaysia
Protocol No : -
MEC Ref. No : 902.5

Dengan hormatnya saya merujuk kepada perkara di atas.

Bersama-sama ini dilampirkan surat pemakluman keputusan Jawatankuasa Etika Perubatan yang bermesyuarat pada 15 Februari 2012 untuk makluman dan tindakan tuan selanjutnya.

2. Sila maklumkan kepada Jawatankuasa Etika Perubatan mengenai butiran kajian samada telah tamat atau diteruskan mengikut jangka masa kajian tersebut.

Sekian, terima kasih.

"BERKHIDMAT UNTUK NEGARA"

Saya yang menurut perintah,

Norashikin Mahmood
Setiausaha
Jawatankuasa Etika Perubatan
Pusat Perubatan Universiti Malaya

s.k Ketua
Jabatan Mikrobiologi Perubatan

JABATAN KUALTI
PUSAT PERUBATAN UNIVERSITI MALAYA
(University Malaya Medical Centre)
LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
☎+603-79493209 (office) :🖷 +603-79494638
www.ummc.edu.my :✉ Info@ummc.edu.my

Leading Healthcare

UNIVERSITI MALAYA
PUSAT PERUBATAN UM

MEDICAL ETHICS COMMITTEE
UNIVERSITY MALAYA MEDICAL CENTRE
ADDRESS: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEPHONE: 03-79493209 FAXIMILE: 03-79494638

NAME OF ETHICS COMMITTEE/IRB: Medical Ethics Committee, University Malaya Medical Centre **ADDRESS:** LEMBAH PANTAI 59100 KUALA LUMPUR	**ETHICS COMMITTEE/IRB REFERENCE NUMBER:** 902.5
PROTOCOL NO: **TITLE:** Multidrug efflux pumps over-expression and porin down- regulation in association with drug resistance among nosocomial pseudomonas aeruginosa isolates in Malaysia	
PRINCIPAL INVESTIGATOR : Dr. Adbelkodose M Al- Kabsi **TELEPHONE:** **KOMTEL:**	**SPONSOR:** UMRG

The following item [✓] have been received and reviewed in connection with the above study to be conducted by the above investigator.

[✓] Application Form	Ver date: 26 Jan 12
[✓] Study Protocol	Ver date:
[ ] Investigator Brochure	Ver date:
[ ] Consent Form	
[ ] Patient Information Sheet	
[ ] Questionnaire	
[✓] Investigator(s) CV's (Dr. Adbelkodose M Al- Kabsi)	

and have been [✓]

[✓] Approved
[ ] Conditionally approved (identify item and specify modification below or in accompanying letter)
[ ] Rejected (identify item and specify reasons below or in accompanying letter)

Comments:

Investigator are required to:

1) *follow instructions, guidelines and requirements of the Medical Ethics Committee.*
2) *report any protocol deviations/violations to Medical Ethics Committee.*
3) *provide annual and closure report to the Medical Ethics Committee.*
4) *comply with International Conference on Harmonization – Guidelines for Good Clinical Practice (ICH-GCP) and Declaration of Helsinki.*
5) *note that Medical Ethics Committee may audit the approved study.*

Date of approval: 15th FEBRUARY 2012

c.c Head
Department of Medical Microbiology

Deputy Dean (Research)
Faculty of Medicine

Secretary
Medical Ethics Committee
University Malaya Medical Centre

.....
PROF. DATUK LOOI LAI MENG
Chairman
Medical Ethics Committee

UNIVERSITI MALAYA
PUSAT PERUBATAN UM

MEDICAL ETHICS COMMITTEE
UNIVERSITY MALAYA MEDICAL CENTRE
ADDRESS: LEMBAH PANTAI, 59100 KUALA LUMPUR, MALAYSIA
TELEPHONE: 03-79493209 FAXIMILE: 03-79494638

MEDICAL ETHICS COMMITTEE COMPOSITION, UNIVERSITY MALAYA MEDICAL CENTRE
Date: 15th FEBRUARY 2012

Member (Title and Name)	Occupation (Designation)	Male/Female (M/F)	Tick (✓) if present when above items were reviewed
Chairperson: Y. Bhg. Prof. Datuk Looi Lai Meng	Senior Consultant Department of Pathology	Female	✓
Deputy Chairperson: Prof. Kulenthran Arumugam	Senior Consultant Medical Education Research and Development Unit (MERDU)	Male	
Secretary (non-voting): Cik Norashikin Mahmood	Scientific Officer Department of Quality	Female	✓
Members: 1. Y. Bhg. Prof. Dato' Patrick Tan Seow Koon	Deputy Director (Professional) University Malaya Medical Centre	Male	
2. Prof. Tan Chong Tin	Representative of Head Department of Medicine	Male	
3. Assoc. Prof. Stephen Thevananthan a/l Jambun	Representative of Head Department of Psychological Medicine	Male	✓
4. Assoc. Prof. Alizan Abdul Khalil	Head Department of Surgery	Male	✓
5. Dr. Poppy Rajan	Representative of Head Department of Pharmacology	Male	✓
6. Pn. Che Zuraini bt. Sulaiman	Representative of Head of Pharmacist Pharmacy Department University Malaya Medical Centre	Female	✓
7. Y. Bhg. Assoc .Prof. Datin Grace Xavier	Representative of Dean (Research Fellow) Faculty of Law University Malaya	Female	✓
8. Y. Bhg. Datin Aminah bt. Pit Abdul Rahman	Public Representative	Female	
9. Madam Ong Eng Lee	Public Representative	Female	✓

Comments: The MEC of University Malaya Medical Centre is operating according to ICH-GCP guidelines and the Declaration of Helsinki. Member's no. 7, 8 & 9 are representatives from Faculty of Law in the University Malaya and the public. They are independent of the hospital or trial site.

.....
PROF. DATUK LOOI LAI MENG
Chairman
Medical Ethics Committee

APPENDIX II

EQUIPMENT AND MATERIALS

1. Electric- Binocular Microscope, CH-30 (Olympus®)
2. Automatic Micropipettes (eppendorf®).
3. Master cycler Gradient PCR (Eppendroff®).
4. Real time CFX96™ PCR (BioRad®, Hercules, CA).
5. Nano drop-2000 spectrophotometer
6. Laminar flow
7. Apparatus for agarose gel electrophoresis (BioRad ®).
8. UV transilluminator, gel doc XR system (BioRad ®, Hercules, CA)
9. Refrigerator (4°C) and freezer (–20°C & –80°C).
10. Centrifuge for 1.5 Microcentrifuge tubes (Eppendroff®).
11. Centrifuge for 50 mL tubes (BioRad®).
12. Microcentrifuge (for 0.2 mL PCR tube).
13. Vortex and shaker.
14. Tips, different sizes; (0.1-10 µL, 0.5-300µL and 300-1000µL) (eppendorf®).
15. 1.5 mL Microcentrifuge tubes (eppendorf®)
16. 0.2 mL PCR tubes (BioRad®).
17. Incubator 37°C (MEMMERT®).
18. Conical centrifuge tube 50 mL
19. Water bath (MEMMERT®).

B- Reagents

B.1 Gram stain reagents

1. Primary stain
   Solution 1: Crystal violet 2 g
   Ethanol 95% 20 mL
   Solution 2: Ammonium oxalate 0.8 g
   Distilled water 100 mL

-Mix Solution 1 and Solution 2, allow to stand for at least 24 hours, and filter before use

2. Gram's iodine:
Potassium iodide 2 g
Iodine crystals 1 g
Distilled water 100 mL

3. Decolorizer: 50 mL acetone added to 50 mL ethanol.

4. Counterstain:
Safranin 4.0 g
Ethanol 95% 200 mL
Distilled water 800 mL
Working solution: Stock solution 10 mL
Distilled water 90 mL

Standard biochemical reagents

Blood agar
Trypton/peptic digest of animal tissue 10 g
Brain heart infusion 500g
Sodium chloride 5g
Agar 15g
Distilled water 1000
- Sterilize by autoclaving.
- Cool media to 45-50°C, then add preheated (45°C) sterile blood

B.2 Preparation of sterile normal saline

8.5 g of NaCl was added to 1.0 L distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes.

B.3 Preparation of sterile PBS

A total number of 5 PBS tablets were dissolved in 500 mL of distilled water and mixed well. The mixture was autoclaved at 121°C for 15 minutes.

B.4 Preparation of 50× TAE buffer

24.2 g of Tris Base was added with 5.71 mL of glacial acetic acid and 10 mL of 0.5 M EDTA (PH 8). The mixture was topped up with distilled water to 100 mL. Finally, the solution was autoclaved at 121°C for 15 minutes.

B.5 10X TBE Buffer (Tris-Borate-EDTA) (Fermentas®):

1X TBE buffer is composed of 89 mM Tris, 89 mM boric acid, 2 mM EDTA, (pH 8.3), Stored at room temperature.

B.6 TE buffer (10 mM Tris·Cl, 1 mM EDTA, pH 8.0) containing 15 mg/mL Lysozyme

1M Tris-HCL., pH 8.0	1mL (FINAL CONCENTRATION = 10 mM)
0.5 EDTA	200 µL (FINAL CONCENTRATION = 1 mM)
Distilled water	100 mL
Lysozyme	150 g

Sterilize by passing through 0.2 Millipore micro filters.

B.7 Ethidium bromide solution:

10 mg/mL in water. Extreme care should be taken during handling this solution as it is highly mutagenic, store in the dark at 4°C.

B.8 Preparation of McFarland standard test

Turbidity standard was prepared by adding 0.5 mL of 0.048 mol/L $BaCl_2$ (1.175% w/v $BaCl_2.2H_2O$) to 99.5 mL of 0.18 mol/L (0.36 N) H_2SO_4 (1% v/v). The density

of mixture was measured at 620 nm using spectrophotometer. The absorbance was 0.080-0.10 at 620 nm. The mixture was stored in the dark at room temperature for up to 4 months.

B.9 Formaldehyde agarose denaturing gel electrophoresis

1. 1.2% agarose gel was prepared by mixing 1.5 mL of 10× formaldehyde gel buffer with DEPC-treated water to 15 mL and 0.18 g agarose in a conical flask and microwave until dissolved. The mixture was let cooled slightly and 0.5 µL of ethidium bromide and 1.8 µL of 37% formaldehyde was added. The gel was then poured in the gel cast and left to set. The gel was then soaked in 1× formaldehyde gel running buffer for at least 30 minutes to equilibrate.
2. 6 µL of DNase treated RNA was mixed with 1 µL of RNA loading dye.
3. The mixture was then heated in a heat block at 65°C for 4 minutes.
4. After heating, the mixture was chilled immediately on ice for 3 minutes.
5. The mixture was then loaded into the formaldehyde gel. The gel electrophoresis was done at 70 V for 40 minutes.
6. The gel was then viewed using UV Transilluminator

B. 10 Preparation of 100 bp DNA ladder

10 µL of 100 bp DNA ladder stock solution was added to 10 µL of 6× loading dye and 40 µL of sterile ultrapure water. Finally, the mixture was gently vortexed and centrifuged.

B.11 Preparation of 2.0% Agarose Gel

2.0 g of agarose powder was added to 100 mL of 1× TAE buffer and mixed gently. The mixture was boiled in a microwave for 3 min until dissolved completely. Liquid mixture was cooled and then 0.8 µL (10 mg/mL) of ethidium bromide was added and swirled gently.

APPENDIX III Raw data supplement

API® 20NE Result for *P. aeruginosa* strains

Test	Strains																																																										
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51	52	53	54	55	56	57	58	59	60
Reduction of Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Iodole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose acidification	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine Dihydrolase	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Urease	-	+	+	+	+	-	+	+	+	-	+	-	-	+	+	-	-	+	-	+	-	+	+	-	-	-	+	-	-	+	-	-	+	+	-	-	+	-	-	-	-	-	+	+	+	+	-	+	+	-	+	+	+	+	-	-	+	+	+	+
Esculin Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	-	-	-	+	+	+	+	+	+	+	-	+	-	+	-	+	-	+	+
Gelatine Hydrolysis	+	+	+	+	+	+	+	+	+	+	+	+	-	+	-	-	-	+	-	+	+	+	+	+	-	-	-	+	+	+	-	-	+	+	-	-	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+
B-galactosidase	+	+	+	+	+	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	=	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose assimilation	-	-	-	-	-	-	+	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-Glucosamine assimilation	+	+	+	+	+	+	+	+	+	-	-	-	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Maltose assimilation	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caprate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adipate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenyl-acetate assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

APPENDIX III Raw data supplement

API® 20NE Result for (*P. aeruginosa)* strains

Test	Strains																												
	61	62	63	64	65	66	67	68	69	70	71	72	73	74	75	76	77	78	79	80	81	82	83	84	85	86	87	88	ATCC
Reduction of Nitrate	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Iodole production	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose acidification	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Arginine Dihydrolase	+	+	-	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+	+
Urease	+	-	-	-	-	-	+	+	+	+	-	-	+	+	+	+	-	-	-	+	-	+	+	+	+	-	-	-	+
Esculin Hydrolysis	+	+	+	-	-	-	+	-	+	-	+	-	+	-	+	+	+	+	-	+	-	+	-	+	+	+	+	-	+
Gelatine Hydrolysis	+	+	+	-	+	-	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-	+
B-galactosidase	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Glucose assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Arabinose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Mannitol assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
N-Acetyl-Glucosamine assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	+	+	-
Maltose assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Gluconate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Caprate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Adipate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Malate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Citrate assimilation	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Phenyl-acetate assimilation	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Cytochrome oxidase	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+

APPENDIX III Raw data supplement

Antibiotics susceptibility test

No.	PATIENT REFERENCE No.	Minimum inhibition concentration (MIC µg/mL)										
		WARD	**SOURCE**	**IMP**	**MEP**	**CAZ**	**AZT**	**PPT**	**GN**	**AK**	**CIP**	**CL**
#	PS 27853	-	-	1.50	0.50	1.50	4.00	4.00	2.00	4.00	0.12	3.00
1	PS/0901-5	ORTHOPEDIC	WOUND	2.00	3.00	256.00	8.00	96.00	1024.00	48.00	32.00	3.00
2	PS/0901-6	NEUROSURGERY	WOUND	32.00	32.00	256.00	32.00	24.00	32.00	16.00	32.00	4.00
3	PS/0901-7	SURGERY	WOUND	32.00	32.00	256.00	8.00	256.00	1024.00	8.00	32.00	3.00
4	PS/0902-1	NEUROSURGERY	T/SUCTION	32.00	32.00	256.00	16.00	24.00	64.00	24.00	32.00	4.00
5	PS/0902-3	SURGERY	URINE	1.50	3.00	96.00	24.00	256.00	192.00	16.00	32.00	4.00
6	PS/0903-2	PAEDITRIC	URINE	32.00	32.00	48.00	16.00	256.00	1024.00	256.00	32.00	3.00
7	PS/0903-3	SURGERY	URINE	32.00	32.00	256.00	256.00	32.00	1024.00	256.00	32.00	6.00
8	PS/0904-2	GYNAECOLOGY	URINE	32.00	32.00	256.00	12.00	256.00	384.00	12.00	16.00	4.00
9	PS/0905-1	SURGERY	WOUND	32.00	32.00	256.00	12.00	128.00	192.00	8.00	32.00	4.00
10	PS/0905-2	ORTHOPEDIC	WOUND	32.00	32.00	256.00	32.00	32.00	48.00	24.00	32.00	6.00
11	PS/0906-1	NEUROSURGERY	URINE	32.00	32.00	256.00	32.00	24.00	48.00	32.00	32.00	4.00
12	PS/0906-2	ORTHOPEDIC	WOUND	32.00	32.00	256.00	48.00	24.00	384.00	256.00	32.00	4.00
13	PS/0811-1	NEUROSURGERY	URINE	32.00	32.00	256.00	24.00	32.00	48.00	32.00	32.00	3.00
14	PS/0811-2	MEDICAL	T/SUCTION	32.00	32.00	256.00	256.00	256.00	1024.00	256.00	32.00	4.00
15	PS/0812-1	SURGERY	URINE	32.00	32.00	256.00	256.00	12.00	1024.00	256.00	32.00	4.00
16	PS/0812-2	SURGERY	URINE	32.00	32.00	256.00	256.00	12.00	1024.00	256.00	32.00	4.00
17	PS/0812-3	PAEDITRIC	CSF	32.00	32.00	32.00	24.00	256.00	8.00	8.00	125.00	4.00
18	PS/0812-4	NEUROSURGERY	SPUTUM	32.00	32.00	256.00	256.00	32.00	1024.00	256.00	32.00	3.00
19	PS/0812-6	SURGERY	URINE	32.00	32.00	256.00	256.00	32.00	1024.00	256.00	32.00	3.00
20	PS/0809-6	ORTHOPEDIC	URINE	32.00	32.00	256.00	48.00	256.00	1024.00	24.00	32.00	2.00
21	PS/0809-7	SURGERY	WOUND	32.00	32.00	256.00	16.00	256.00	1024.00	24.00	32.00	2.00
22	PS/0810-1	SURGERY	URINE	2.00	32.00	256.00	256.00	256.00	384.00	24.00	32.00	2.00
23	PS/0810-2	SURGERY	SPUTUM	2.00	32.00	256.00	64.00	256.00	384.00	16.00	32.00	3.00
24	PS/0810-3	MEDICAL	URINE	32.00	32.00	32.00	48.00	128.00	192.00	8.00	32.00	1.00
25	PS/0810-4	MEDICAL	URINE	32.00	32.00	256.00	12.00	8.00	24.00	16.00	32.00	2.00
26	PS/0811-4	SURGERY	Tissue	1.00	3.00	256.00	4.00	16.00	1024.00	48.00	3.00	2.00
27	PS/0811-5	SURGERY	URINE	32.00	32.00	256.00	64.00	256.00	1024.00	24.00	32.00	2.00

28	PS/0811-6	MEDICAL	URINE	.50	3.00	4.00	16.00	256.00	24.00	4.00	32.00	.25
29	PS/0811-7	SURGERY	URINE	2.00	3.00	256.00	256.00	256.00	1024.00	48.00	32.00	2.00
30	PS/0809-5	ORTHOPEDIC	WOUND	2.00	3.00	24.00	24.00	256.00	1024.00	256.00	32.00	3.00
31	PS/0809-4	MEDICAL	URINE	32.00	32.00	256.00	16.00	16.00	1024.00	64.00	32.00	3.00
32	PS/0809-3	SURGERY	URINE	2.00	3.00	256.00	32.00	256.00	1024.00	256.00	32.00	3.00
33	PS/0809-2	SURGERY	URINE	32.00	32.00	256.00	192.00	256.00	1024.00	32.00	32.00	4.00
34	PS/0809-1	NEUROSURGERY	URINE	1.50	3.00	256.00	256.00	256.00	1024.00	32.00	32.00	2.00
35	PS/0808-8	ORTHOPEDIC	Tissue	32.00	32.00	16.00	256.00	96.00	32.00	126.00	1.50	1.50
36	PS/0808-7	ENT	pus	4.00	1.00	256.00	1.00	1.00	64.00	6.00	32.00	2.00
37	PS/0808-6	SURGERY	WOUND	1.50	32.00	256.00	24.00	128.00	1024.00	192.00	4.00	2.00
38	PS/0808-5	SURGERY	URINE	32.00	32.00	256.00	128.00	256.00	1024.00	48.00	32.00	4.00
39	PS/0808-4	MEDICAL	T/SUCTION	32.00	32.00	256.00	16.00	256.00	96.00	48.00	32.00	1.50
40	PS/0808-3	CICU	T/SUCTION	32.00	32.00	32.00	48.00	256.00	2.00	4.00	.25	1.50
41	PS/0808-2	SURGERY	URINE	32.00	32.00	256.00	256.00	32.00	1024.00	256.00	32.00	3.00
42	PS/0808-1	ORTHOPEDIC	URINE	3.00	3.00	256.00	96.00	48.00	1024.00	256.00	32.00	3.00
43	PS/0807-3	MEDICAL	WOUND	2.00	3.00	256.00	12.00	64.00	1024.00	96.00	3.00	2.00
44	PS/0807-2	SURGERY	URINE	32.00	32.00	256.00	24.00	256.00	1024.00	32.00	32.00	2.00
45	PS/0807-1	SURGERY	URINE	32.00	32.00	256.00	32.00	256.00	1024.00	24.00	32.00	2.00
46	PS/0806-4	SURGERY	T/SUCTION	32.00	32.00	32.00	256.00	256.00	1024.00	256.00	32.00	2.00
47	PS/0806-3	ORTHOPEDIC	URINE	32.00	32.00	32.00	6.00	256.00	1024.00	256.00	32.00	2.00
48	PS/0806-1	PAEDITRIC	URINE	32.00	32.00	192.00	192.00	256.00	1024.00	256.00	32.00	1.00
49	PS/0806-2	GYNAECOLOGY	URINE	32.00	32.00	256.00	256.00	96.00	1024.00	192.00	32.00	2.00
50	PS/0805-7	SURGERY	URINE	32.00	32.00	256.00	32.00	256.00	1024.00	16.00	32.00	3.00
51	PS/0805-6	MEDICAL	URINE	32.00	32.00	256.00	192.00	256.00	1024.00	24.00	32.00	1.00
52	PS/0805-5	ICU	URINE	32.00	32.00	256.00	256.00	256.00	1024.00	24.00	32.00	1.00
53	PS/0805-4	SURGERY	URINE	32.00	32.00	256.00	64.00	256.00	1024.00	24.00	32.00	2.00
54	PS/0805-3	MEDICAL	URINE	32.00	32.00	64.00	256.00	256.00	1024.00	16.00	8.00	1.50
55	PS/0805-2	ICU	lumen tip	32.00	32.00	256.00	256.00	16.00	1024.00	256.00	32.00	4.00
56	PS/0805-1	ICU	WOUND	32.00	32.00	256.00	32.00	256.00	6.00	8.00	.25	3.00
57	PS/0804-3	MEDICAL	URINE	1.50	3.00	256.00	64.00	256.00	1024.00	32.00	32.00	3.00
58	PS/0804-2	ENT	WOUND	32.00	32.00	256.00	1.00	1.00	32.00	48.00	1.50	3.00

59	PS/0804-1	ORTHOPEDIC	WOUND	1.50	3.00	12.00	24.00	256.00	1024.00	256.00	32.00	3.00
60	PS/0803-6	SURGERY	URINE	32.00	32.00	256.00	256.00	24.00	1024.00	256.00	32.00	3.00
61	PS/0803-5	SURGERY	Catheter tip	32.00	32.00	24.00	6.00	256.00	1024.00	256.00	32.00	4.00
62	PS/0803-4	PAEDITRIC	peritoneal fluid	32.00	32.00	3.00	256.00	256.00	32.00	64.00	1.50	48.00
63	PS/0803-3	SURGERY	URINE	32.00	32.00	256.00	256.00	24.00	1024.00	256.00	32.00	4.00
64	PS/0803-2	SURGERY	URINE	32.00	32.00	256.00	24.00	16.00	48.00	32.00	32.00	3.00
65	PS/0803-1	MEDICAL	SPUTUM	32.00	32.00	64.00	6.00	256.00	1024.00	64.00	32.00	3.00
66	PS/0802-6	MEDICAL	Blood	32.00	32.00	64.00	24.00	256.00	1024.00	256.00	32.00	4.00
67	PS/0802-5	ORTHOPEDIC	WOUND	32.00	32.00	256.00	8.00	256.00	1024.00	256.00	32.00	3.00
68	PS/0802-4	MEDICAL	URINE	32.00	32.00	256.00	16.00	256.00	1024.00	256.00	32.00	1.50
69	PS/0909-1	NEUROSURGERY	URINE	12.00	32.00	256.00	8.00	16.00	96.00	32.00	32.00	4.00
70	PS/0909-2	MEDICAL	WOUND	32.00	32.00	48.00	16.00	256.00	1024.00	256.00	32.00	3.00
71	PS/0909-3	SURGERY	Blood	1.00	3.00	256.00	16.00	64.00	1024.00	64.00	6.00	1.50
72	PS/0910-4	ORTHOPEDIC	WOUND	32.00	32.00	256.00	32.00	24.00	256.00	96.00	32.00	3.00
73	PS/0910-3	MEDICAL	URINE	32.00	32.00	256.00	32.00	24.00	1024.00	8.00	32.00	2.00
74	PS/0910-2	ENT	URINE	32.00	3.00	256.00	16.00	96.00	1024.00	96.00	4.00	1.50
75	PS/0910-1	SURGERY	SPUTUM	32.00	32.00	256.00	16.00	96.00	1024.00	96.00	4.00	2.00
76	PS/0911-4	ORTHOPEDIC	Blood	32.00	32.00	256.00	48.00	256.00	1024.00	256.00	32.00	3.00
77	PS/0911-2	PAEDITRIC	URINE	32.00	32.00	256.00	12.00	256.00	1024.00	128.00	32.00	2.00
78	PS/0911-1	MEDICAL	URINE	32.00	3.00	256.00	32.00	96.00	1024.00	3.00	32.00	1.00
79	PS/0909-6	PAEDITRIC	Blood	1.50	1.00	256.00	16.00	256.00	1024.00	98.00	12.00	1.50
80	PS/0909-5	SURGERY	WOUND	32.00	32.00	256.00	256.00	256.00	1024.00	12.00	32.00	6.00
81	PS/0909-4	PAEDITRIC	WOUND	3.00	1.50	256.00	16.00	128.00	1024.00	128.00	16.00	6.00
82	PS/0908-3	MEDICAL	URINE	3.00	32.00	256.00	32.00	48.00	1024.00	256.00	32.00	4.00
83	PS/0908-2	ORTHOPEDIC	WOUND	32.00	32.00	32.00	8.00	256.00	1024.00	256.00	32.00	4.00
84	PS/0907-4	ICU	URINE	32.00	32.00	256.00	256.00	64.00	1024.00	256.00	32.00	2.00
85	PS/0907-3	MEDICAL	URINE	3.00	3.00	16.00	0.25	0.94	1.00	0.64	32.00	2.00
86	PS/0907-2	MEDICAL	SPUTUM	32.00	32.00	256.00	256.00	256.00	8.00	3.00	1.00	.50
87	PS/0907-1	SURGERY	URINE	32.00	32.00	256.00	256.00	24.00	1024.00	256.00	32.00	12.00
88	PS/0906-4	MEDICAL	Blood	32.00	32.00	48.00	16.00	256.00	1024.00	256.00	32.00	4.00

Abbreviations of antibiotics: IMP= imipenem ($S \leq 4, I = 8, R \geq 16$), MPM= meropenem ($S \leq 4, I = 8, R \geq 16$), CAZ= ceftazidime ($S \leq 8, I = 16, R \geq 32$), AZT= aztreonam ($S \leq 8, I = 16, R \geq 32$), PPT= piperacillin/tazobactam ($S \leq 16, I = 32/64, R \geq 128$), GN= gentamicin ($S \leq 4, I = 5, R \geq 8$), AK= amikacin ($S \leq 16, I = 17, R \geq 32$), CIP=ciprofloxacin ($S \leq 1, I = 2, R \geq 4$) AND CL= colistin ($S \leq 2, I = 4, R \geq 8$). PS: (*P. aeruginosa*). MIC= minimum inhibition concentration break point for *P. aeruginosa* according to CLSI (Wikler,2008).

APPENDIX III Raw data supplement

No.			mRNA expression level (fold)									
	WARD	**SOURCE**	***MexB***	***MexY***	***MexZ***	***MexCD***	***MexEF***	***AmpC***	***OprD***	***OprM***	***PBP2***	***PBP3***
#	*P. aeruginosa*	ATCC 27583	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
1	ORTHOPEDIC	WOUND	2.84	18.07	4.21	4.64	2.23	.56	.50	.18	.31	.50
2	NEUROSURGERY	WOUND	2.13	.42	.45	482	51.60	12.19	.41	.20	.21	.71
3	SURGERY	WOUND	0.80	4.46	.31	6.13	4.25	19.03	.11	.07	.08	.57
4	NEUROSURGERY	T/SUCTION	2.88	4.31	.42	2.56	3.25	.61	.33	.20	.26	.05
5	SURGERY	URINE	4.10	40.50	7.29	.42	.23	632	1.28	2.01	.40	.25
6	PAEDITRIC	URINE	2.41	25.48	4.73	4.37	.63	12.94	.47	.36	.00	.19
7	SURGERY	URINE	2.61	8.87	2.31	.50	.30	.64	.27	.46	.40	.17
8	GYNAECOLOGY	URINE	0.51	.83	.26	.34	.73	14.92	.22	.07	.22	.31
9	SURGERY	WOUND	3.44	21.62	3.88	.98	2.75	13.41	.27	.35	.82	.75
10	ORTHOPEDIC	WOUND	2.03	.33	.46	2.43	2.06	.60	.31	.32	.55	.03
11	NEUROSURGERY	URINE	11.50	4.44	.42	15.75	23.91	.63	.46	.35	.58	.15
12	ORTHOPEDIC	WOUND	4.26	11.12	3.10	4.37	11.13	.52	.46	.35	.61	.67
13	NEUROSURGERY	URINE	2.30	10.31	5.70	2.43	3.15	18.54	.74	.51	.18	.84
14	MEDICAL	T/SUCTION	2.62	43.49	3.77	3.35	40.41	.99	.25	.18	.22	.49
15	SURGERY	URINE	2.63	7.06	2.69	19.98	159.7	.44	.23	.33	.23	.08
16	SURGERY	URINE	2.23	4.28	2.04	2.50	2.35	.26	.18	.17	.06	.04
17	PAEDITRIC	CSF	3.60	4.49	4.77	2.40	2.96	.84	.57	.49	.57	.33
18	NEUROSURGERY	SPUTUM	2.40	5.79	2.60	5.24	9.58	17.58	.49	3.75	.41	.07
19	SURGERY	URINE	2.07	4.99	2.04	22.18	15.27	.26	.21	.19	.07	.02
20	ORTHOPEDIC	URINE	2.20	5.68	.02	2.00	4.23	16.69	.13	.28	.12	.41

21	SURGERY	WOUND	.55	7.78	2.84	2.90	2.00	13.75	.19	.14	.20	.09
22	SURGERY	URINE	6.98	4.61	7.43	7.50	12.03	12.27	.49	3.68	1.51	.21
23	SURGERY	SPUTUM	3.84	7.01	8.22	3.89	52.44	12.81	.20	2.47	5.24	.31
24	MEDICAL	URINE	4.30	4.58	4.67	6.80	8.43	.09	.37	6.73	3.25	.53
25	MEDICAL	URINE	2.17	.02	6.38	52.69	2.93	12.99	.47	2.53	19.88	.16
26	SURGERY	Tissue	2.44	49.14	4.88	2.91	4.42	.61	.43	.53	.54	.66
27	SURGERY	URINE	3.42	13.85	5.27	.36	2.89	28.92	.48	.67	.44	.37
28	MEDICAL	URINE	6.14	15.81	2.83	5.24	7.93	12.03	1.07	.64	.28	.25
29	SURGERY	URINE	2.40	1731	408	.05	2.29	12.99	.56	.48	1.10	4.13
30	ORTHOPEDIC	WOUND	2.50	8.81	.10	22.19	9.18	19.58	.10	3.14	.20	.42
31	MEDICAL	URINE	0.74	4.34	.74	2.85	6.18	.58	.12	.72	.30	.48
32	SURGERY	URINE	50.06	410.0	7.52	.35	.40	15.23	3.54	18.59	3.73	6.37
33	SURGERY	URINE	4.67	69.49	4.63	5.28	2.96	147.0	.12	.58	.67	.08
34	NEUROSURGERY	URINE	2.47	83.16	6.82	.60	.20	.22	.17	3.30	.10	.14
35	ORTHOPEDIC	Tissue	2.70	20.43	2.84	.51	.42	13.71	.28	7.49	.09	.05
36	ENT	pus	11.60	9.47	4.73	.20	.11	16.21	.69	12.47	.83	.54
37	SURGERY	WOUND	2.14	10.56	2.13	2.99	7.55	.17	.11	.70	.25	.26
38	SURGERY	URINE	20.82	45.61	14.52	2.03	2.62	102.2	.08	11.10	.30	.44
39	MEDICAL	T/SUCTION	10.98	4.53	.15	2.47	3.52	114.6	.36	7.10	.38	.37
40	CICU	T/SUCTION	.23	.18	.50	.10	.42	237.4	.16	.41	.15	.15
41	SURGERY	URINE	3.81	52.99	.43	.36	.66	14.92	.83	3.63	.50	.28
42	ORTHOPEDIC	URINE	0.56	13.97	.05	.48	.72	13.41	.58	1.06	.34	.14
43	MEDICAL	WOUND	0.26	5.86	.15	.40	.70	16.94	.17	.22	.08	.02
44	SURGERY	URINE	0.46	4.11	.02	.38	.27	17.00	.32	.44	.33	.26
45	SURGERY	URINE	0.37	8.71	.00	2.45	.00	14.50	.19	.45	.51	.04
46	SURGERY	T/SUCTION	.49	16.78	.26	.78	2.10	19.70	.41	.38	.13	.04
47	ORTHOPEDIC	URINE	.26	7.92	.21	.00	.00	13.70	.14	.15	.82	.04
48	PAEDITRIC	URINE	2.50	23.48	.30	.94	.07	12.90	.28	1.16	.16	.04

49	GYNAECOLOGY	URINE	0.73	.00	.05	.82	.40	11.40	.36	.65	.12	.06
50	SURGERY	URINE	2.77	8.51	2.48	4.88	2.21	18.64	.14	.29	.14	.08
51	MEDICAL	URINE	2.30	4.74	2.85	9.33	6.77	12.85	.73	.53	.48	.17
52	ICU	URINE	4.90	.04	3.86	4.33	6.23	15.82	.26	.77	2.78	.05
53	SURGERY	URINE	0.64	25.99	.62	.38	.39	13.29	.42	.22	.08	.13
54	MEDICAL	URINE	8.01	32.54	2.70	2.43	2.35	1806	.46	3.71	.12	.13
55	ICU	lumen tip	6.29	13.19	4.16	2.25	2.96	12.99	.40	.03	.69	.13
56	ICU	WOUND	5.08	.00	5.94	.03	.11	14.37	.07	.74	.89	1.12
57	MEDICAL	URINE	3.33	31.84	2.23	6.69	54.51	.43	.17	2.41	.30	.37
58	ENT	WOUND	6.80	4.75	4.87	11.40	13.55	.82	.21	2.71	1.62	.67
59	ORTHOPEDIC	WOUND	3.95	4.31	3.48	.09	.22	16.13	.31	.41	.74	.41
60	SURGERY	URINE	.50	.37	.58	8.44	3.58	10.41	.04	.39	.63	.27
61	SURGERY	Catheter tip	0.03	5.75	.10	2.14	2.73	15.74	.07	.13	.19	.03
62	PAEDITRIC	peritoneal fluid	0.65	105	314	858	371	12.67	.07	.02	1.57	3.91
63	SURGERY	URINE	0.61	35.54	.32	2.68	2.90	12.63	.63	.57	.45	.15
64	SURGERY	URINE	0.01	29.89	.32	.51	.25	17.12	.35	2.67	.37	.12
65	MEDICAL	SPUTUM	2.32	6.84	.25	2.78	2.06	12.26	.11	.04	.08	.15
66	MEDICAL	Blood	2.33	20.89	.99	.88	2.00	.29	.03	4.55	.22	.18
67	ORTHOPEDIC	WOUND	2.33	27.31	2.97	72.04	4.86	.54	.07	3.82	.67	.60
68	MEDICAL	URINE	5.88	74.39	3.67	177.6	165.6	.63	.18	1.52	.55	.47
69	NEUROSURGERY	URINE	6.81	230.8	8.95	4.02	10.13	16.94	.93	1.21	.83	1.38
70	MEDICAL	WOUND	4.73	279.8	8.94	28.18	21.07	16.87	.67	.99	.35	1.49
71	SURGERY	Blood	2.06	4.95	8.38	.23	.12	.88	.15	.44	.35	1.90
72	ORTHOPEDIC	WOUND	9.86	123.1	8.96	5.20	4.78	12.86	.47	2.34	1.56	.97
73	MEDICAL	URINE	4.32	24.63	3.87	3.26	6.14	.20	.45	2.52	.94	.59
74	ENT	URINE	38.37	.49	2.91	.31	.54	12.40	.41	1.06	1.14	1.42
75	SURGERY	SPUTUM	102	.54	2.03	522	3.26	.81	.83	5.15	.05	9.76
76	ORTHOPEDIC	Blood	.35	.59	2.62	2.80	4.20	.53	.05	.18	1.18	.54

77	PAEDITRIC	URINE	3.72	4.76	2.55	4.78	18.13	17.56	.53	1.29	3.28	.42
78	MEDICAL	URINE	2.15	123	272.8	.14	.23	12.37	.11	.47	4.12	3.12
79	PAEDITRIC	Blood	.39	4.40	14.33	.47	.73	.21	.06	.73	.46	.90
80	SURGERY	WOUND	17.10	.01	5.03	7.30	12.30	.18	.57	1.89	4.67	2.57
81	PAEDITRIC	WOUND	4.81	14.35	15.64	.72	.32	12.13	.40	.67	2.09	1.61
82	MEDICAL	URINE	.03	.35	.63	207.1	130.7	.82	.02	.17	.40	.45
83	ORTHOPEDIC	WOUND	5.24	29.52	6.64	11.81	2.96	73.88	.26	.69	4.91	.32
84	ICU	URINE	2.05	821.7	914	2.41	7.92	.95	.16	.85	1.99	.56
85	MEDICAL	URINE	.47	.64	.74	.64	.51	.01	.64	.29	3.62	2.30
86	MEDICAL	SPUTUM	.69	.73	.58	16.37	19.42	.16	.16	.71	.82	.01
87	SURGERY	URINE	.61	222.5	137	2.75	15.89	13.78	.04	.54	.39	.69
88	MEDICAL	Blood	2.27	329.2	947	14.49	8.79	12.56	.02	.52	2.66	1.21

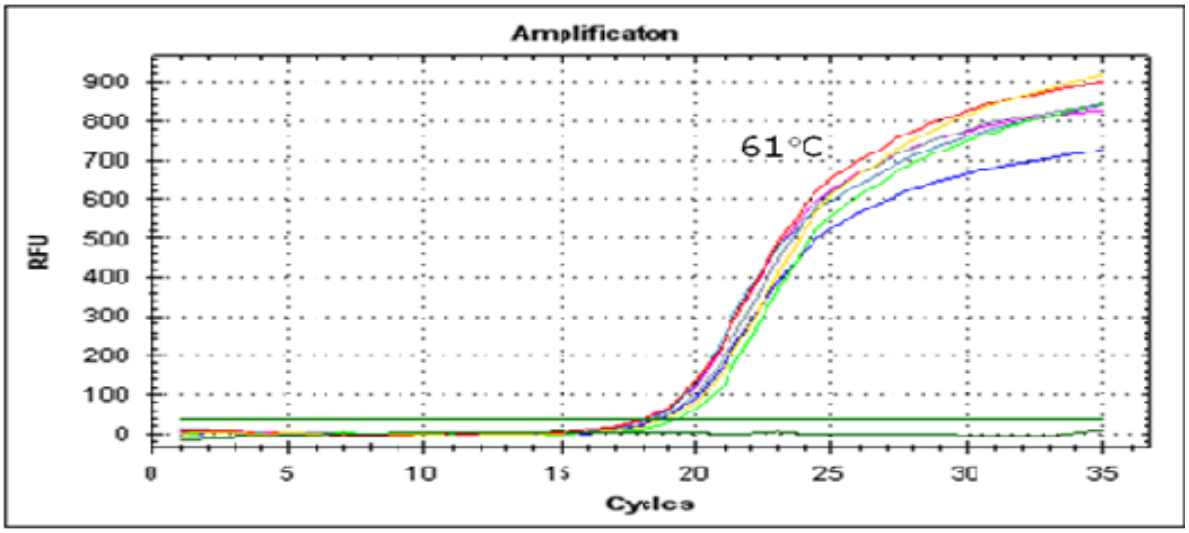
The mRNA transcription levels of gene expression of multidrug resistant isolates in comparison to *P. aeruginosa* ATCC 27583 (n=88)

APPENDIX IV

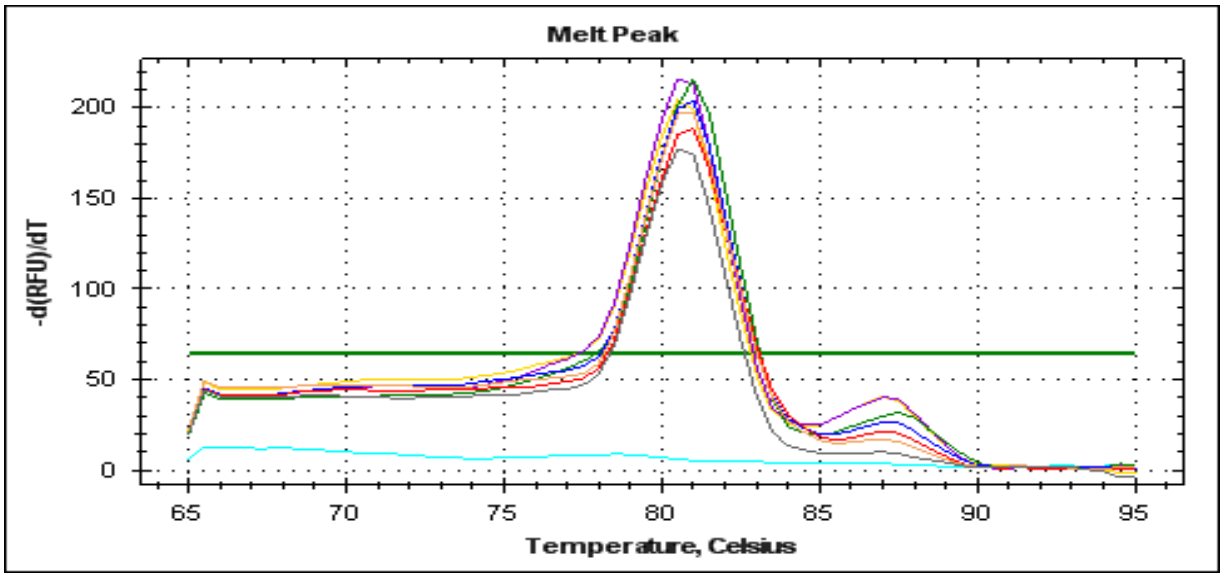
PCR amplification of genes

1. Gradient qRT-PCR *oprD* gene

1.1 Amplification conditions

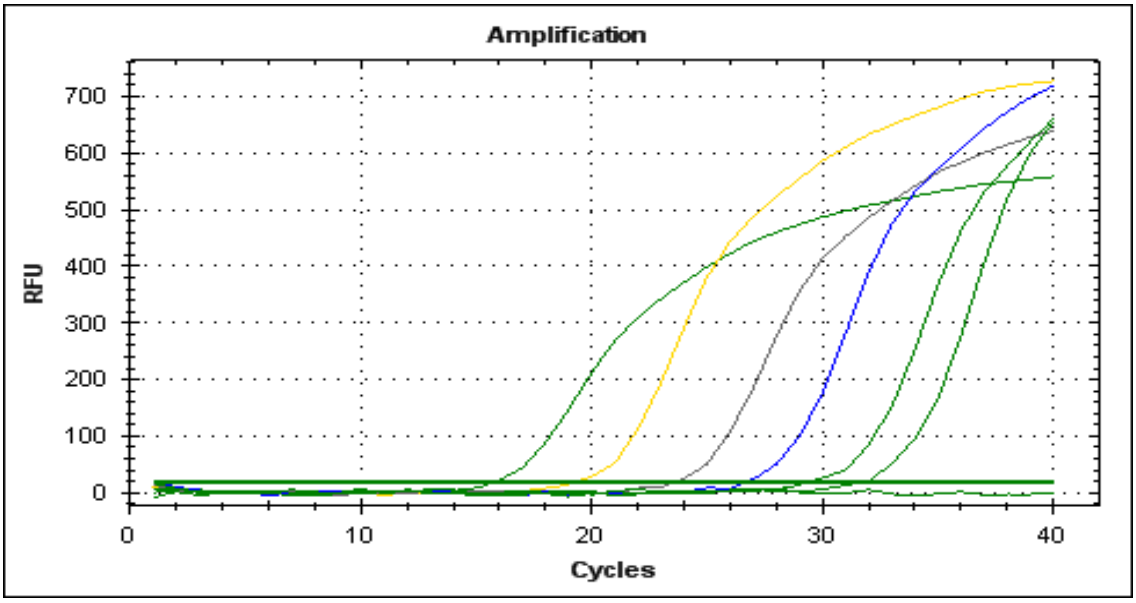


1.1 Melting curve

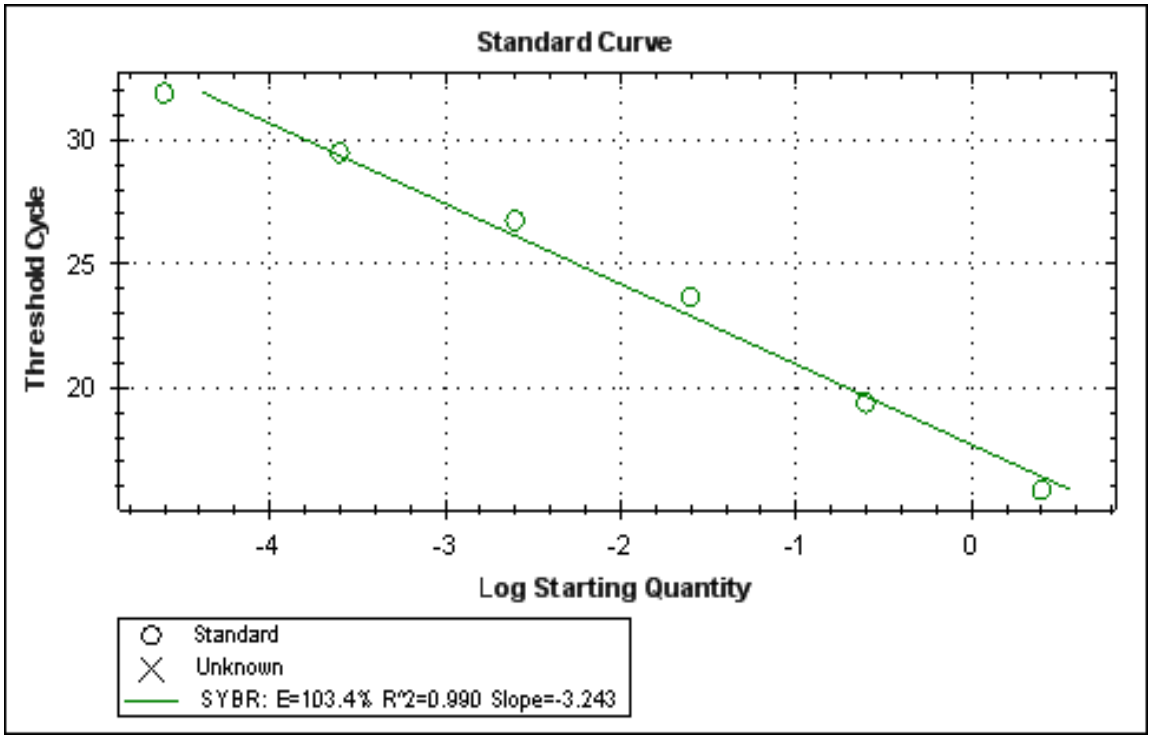


2. Standard curve of qRT-PCR *oprD* gene

2.1 Amplification conditions

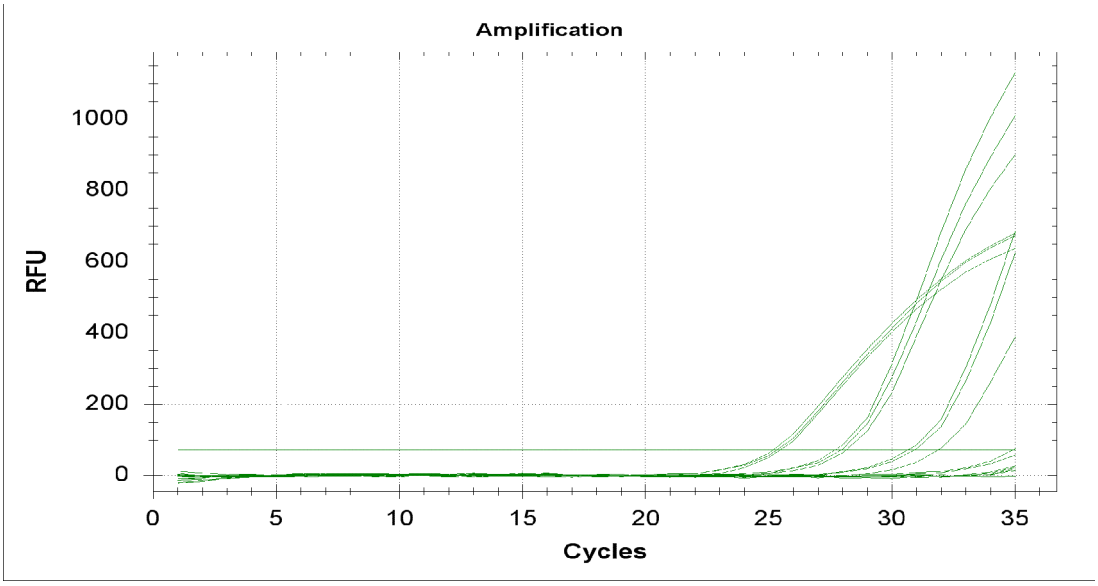


2.2 Standard curve

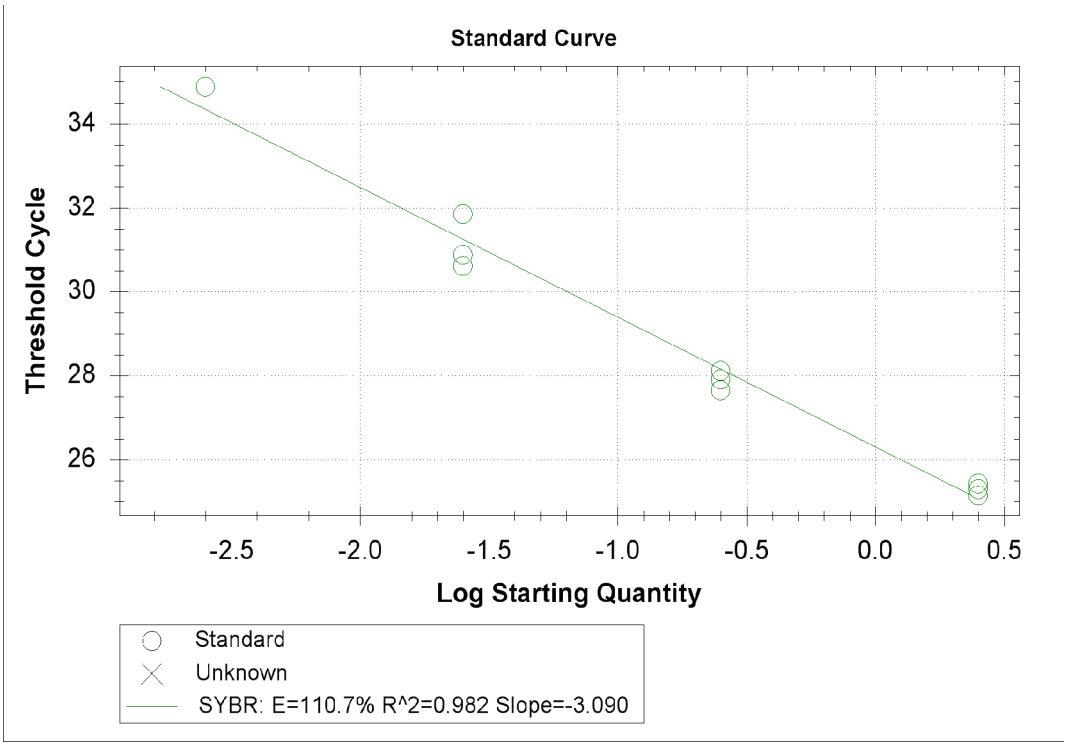


3. Standard curve of qRT-PCR *mexB* gene

3.1 Amplification conditions

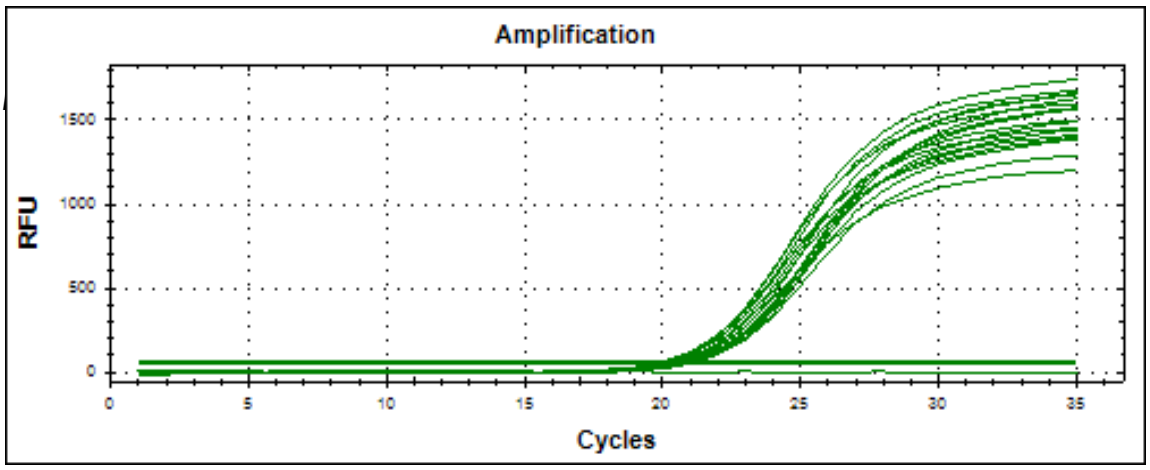


3.2 Standard curve

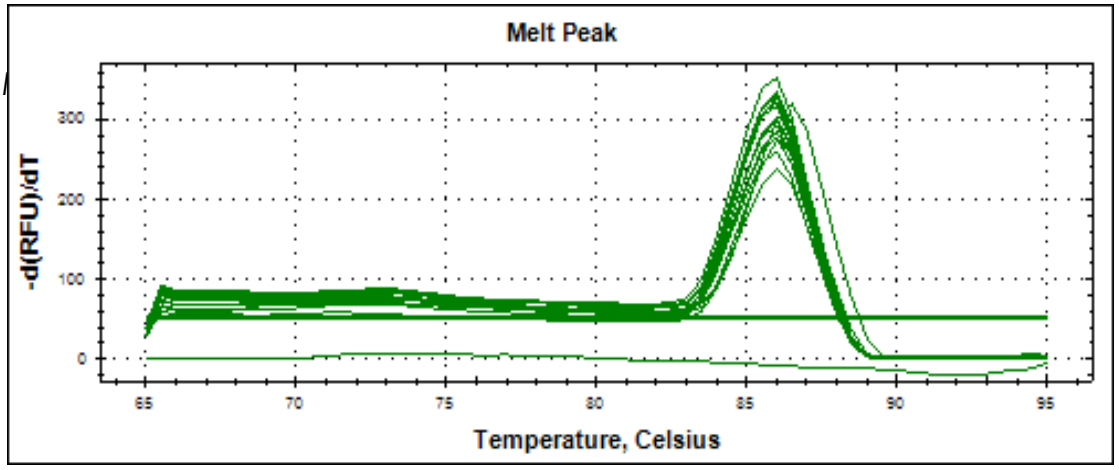


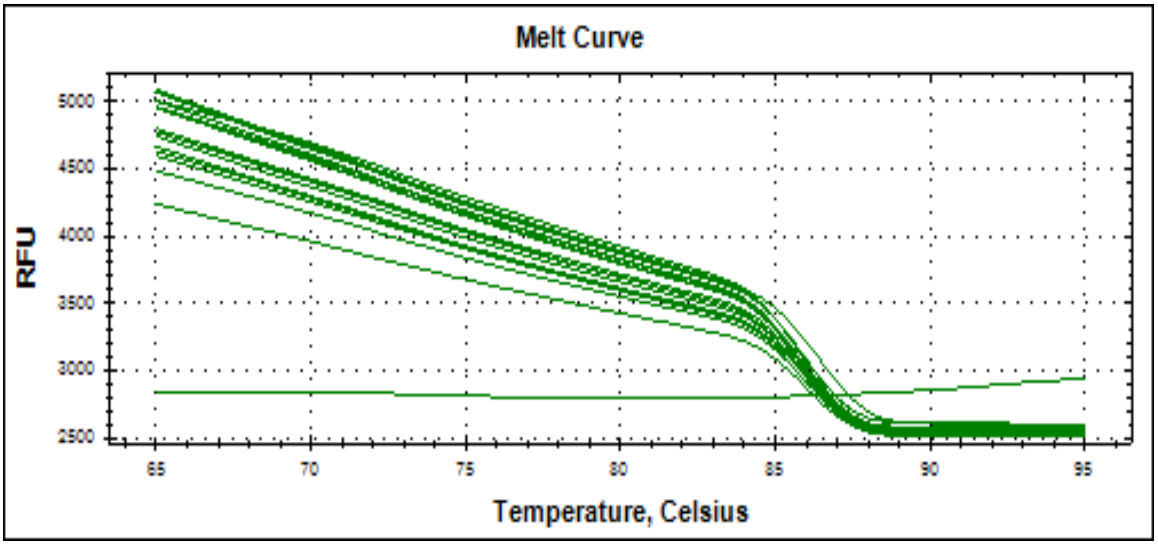
4 Reference gene (*rpoD*)

4.1 Amplification conditions



.4.2 Amplification conditions





Reresnative examples for the statistical analysis (Chi and Fisher's exact test)

Crosstabs

Notes

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
GENTA1 * MEXY1	88	91.7%	8	8.3%	96	100.0%

GENTA1 * MEXY1 Cross tabulation

Count

		MEXY1		
		down	up	Total
GENTA1	S	2	0	2
	I	2	1	3
	R	12	71	83
Total		16	72	88

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)
Pearson Chi-Square	14.515(a)	2	.001
Likelihood Ratio	11.040	2	.004
Linear-by-Linear Association	14.221	1	.000
N of Valid Cases	88		

a 4 cells (66.7%) have expected count less than 5. The minimum expected count is .36.

GENTA1: GENTAMICIN

Crosstabs

Notes

Case Processing Summary

	Cases					
	Valid		Missing		Total	
	N	Percent	N	Percent	N	Percent
IMIP1 * PBP31	88	91.7%	8	8.3%	96	100.0%
IMIP1 * OPRD1	88	91.7%	8	8.3%	96	100.0%

IMIP1 * PBP31

Crosstab

Count

		PBP31		
		down	up	Total
IMIP1	S	16	7	23
	R	58	7	65
Total		74	14	88

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	4.911(b)	1	.027		
Continuity Correction(a)	3.551	1	.060		
Likelihood Ratio	4.433	1	.035		
Fisher's Exact Test				.043	.034
Linear-by-Linear Association	4.855	1	.028		
N of Valid Cases	88				

a Computed only for a 2x2 table

b 1 cells (25.0%) have expected count less than 5. The minimum expected count is 3.66.

IMIP1: IMIPENEM

IMIP1* OPRD1

Crosstab

Count

		OPRD1		
		down	up	Total
IMIP1	S	20	3	23
	R	65	0	65
Total		85	3	88

Chi-Square Tests

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	8.777(b)	1	.003		
Continuity Correction(a)	5.263	1	.022		
Likelihood Ratio	8.357	1	.004		
Fisher's Exact Test				.016	.016
Linear-by-Linear Association	8.678	1	.003		
N of Valid Cases	88				

a Computed only for a 2x2 table

b 2 cells (50.0%) have expected count less than 5. The minimum expected count is .78.

IMIP1: IMIPENEM

Appendix v

PRESENTATIONS AND PUBLICATIONS

a- PUBLICATIONS

1- Al-Kabsi, A. M., Yusof, M. Y. B. M.andSekaran, S. D. (2011). Antimicrobial resistance pattern of clinical isolate of *P. aeruginosa* in the university of malaya medical center, malaysia. African Journal of Microbiology Research; **5:** 5266-5272. (ISI/SCOPUS Cited Publication).

2- Al-Kabsi, A. M, Mohd Yasim Bin Md Yusof, Marzida Mansor, Gracie Ong SiokYan, Shamala Devi Sekaran. Multidrug efflux pumps over-expression and porin down-regulation in association with drug resistance among nosocomial *P. aeruginosa* isolates in Malaysia. Submitted to International Journal of Medical Sciences, Q1, IF: 2.2 (Revised Manuscript Re submited).

3- Al-Kabsi, A. M, Mohd Yasim Bin Md Yusof, Shamala Devi Sekaran. Characterization of multidrug-resistant and detection of metallo-beta-lactamase-producing among nosocomial *P. aeruginosa* isolates at University of Malaya Medical Center, Malaysia. Submitted to International Journal of molecular Sciences (Under Review).

b- PROCEEDINGS AND PRESENTATION

1. Alkabsi, A. M., Bin, M. Y., Yusof, M.andDevi, S. (2011). Analysis of the antimicrobial resistance gene expression in *P. aeruginosa* clinical isolate by quantitative real-time-pcr in malaysia. Trop. Med. Int. Health; **16:** 267-267. As proceeding for the 7th European Congress on Tropical Medicine & International Health. 3-6 October 2011.

2. (1st AMDI-International Biohealth Science Conference (IBSC) - 2010 Infectious Diseases–Current Challenges" 29th Nov- 1st Dec2010. Antimicrobial resistance pattern of clinical isolate *P. aeruginosa* In UMMC. Oral presentation.

3. National Postgraduate Seminar (NPS) 2012 university of malaya, KUALA LUMPUR "Microbes: Diversity in life, diversity in learning, connecting disciplines" 11TH JULY 2012. Poster presentation.

C- Workshop attended:

1- Basic principle and application of Real Time PCR -CFX96 Real Time PCR system, held on 10th February 2010.

2- Course of Real Time PCR which held on 23rd June 2010.

3- Scientific writing workshop organized by Medical Education and research development unite (MERDU), University of Malaya on 26th -28th July 2011.

4- Biosafety training workshop organized by Institute of Graduate Studies, University of Malaya on 19-20 October 2011.

African Journal of Microbiology Research Vol. 5(29), pp. 5266-5272, 9 December, 2011
Available online at http://www.academicjournals.org/AJMR
ISSN 1996-0808 ©2011 Academic Journals
DOI: 10.5897/AJMR11.284

Full Length Research Paper

Antimicrobial resistance pattern of clinical isolate of *Pseudomonas aeruginosa* in the University of Malaya Medical Center, Malaysia

Abdelkodose M. Al-Kabsi*, Mohd Yasim Bin Md Yusof and Shamala Devi Sekaran

Department of Medical Microbiology, Faculty of Medicine, University of Malaya, Kuala Lumpur 50603, Malaysia.

Accepted 17 November, 2011

Pseudomonas aeruginosa **is considered as one of the leading causes of nosocomial infections. The start of antimicrobial therapy is often empirical and selective pressure on panel of antibiotics; therefore, it is important to know the susceptibility pattern of pathogens in order to select the most appropriate antibiotic. The aim of the current study is to update the rational empirical antimicrobial therapy recommendations. Antimicrobial resistance was done using the E-test method. Urine and wound swab samples were the highest encountered isolates; results were interpreted according to National Committee for Clinical Laboratory Standards guidelines. A total of 88 clinical isolates of *P. aeruginosa* were collected randomly from April 2009 to March 2010 from the University of Malaya Medical Center. *P. aeruginosa* isolated from various clinical samples has lost susceptibility and showed increasing resistance to Gentamicin with 94.3%, followed by (ciprofloxacin) 92%, (ceftazidime) 89.8%, (imipenem) 73.9%, Pipracilline/tazobactam 61.4%, (aztreonam) 52.3%, and (amikacin) 50% and only susceptible to colistin with 92%. In conclusion, most of the isolates showed high levels of resistance to examined antibiotics except colistin and this may indicate the importance of antibiotic susceptibility testing and optimal treatment by combination of drugs.**

Key words: *Pseudomonas aeruginosa*, antimicrobial resistance, nosocomial infections, Malaysia.

INTRODUCTION

Pseudomonas aeruginosa is considered as one of the leading nosocomial pathogens worldwide (Strateva and Yordanov, 2009). It is mostly the causes of morbidity and mortality cases. A problem is made worse when nosocomial pathogens acquire antibiotic resistance (Lagamayo, 2008). The emergence of this organism has a significant impact on treatment outcomes and poses a challenge to the provision of health care and cost-effectiveness.

Unfortunately, the resistance to antipseudomonal agents is on the rise (Rubin et al., 2008). *P. aeruginosa* is known to readily develop multi-drug resistance to various classes of antimicrobial agents, the extensive use of antimicrobial agents and the evolutionary antimicrobial resistance strategies of bacteria have resulted in the emergence of pan-drug resistant bacteriathat is, bacteria with evidence resistance against antipseudomonal penicillin's, cephalosporin's, carbapenem, monobactam, aminoglycosides, fluroquinolone and polymyxins (Babic et al., 2006). In this regard, the current study aims to update the rational empirical antimicrobial therapy recommendation. *P. aeruginosa* isolates were considered to be multidrug resistant if the isolate was resistant to at least three of the following eight drugs: Pipracilline/ tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem and colistin. These agents were selected as representatives of the primary

*Corresponding author. E-mail: yasim@um.edu.my. Tel: 03-79492152.

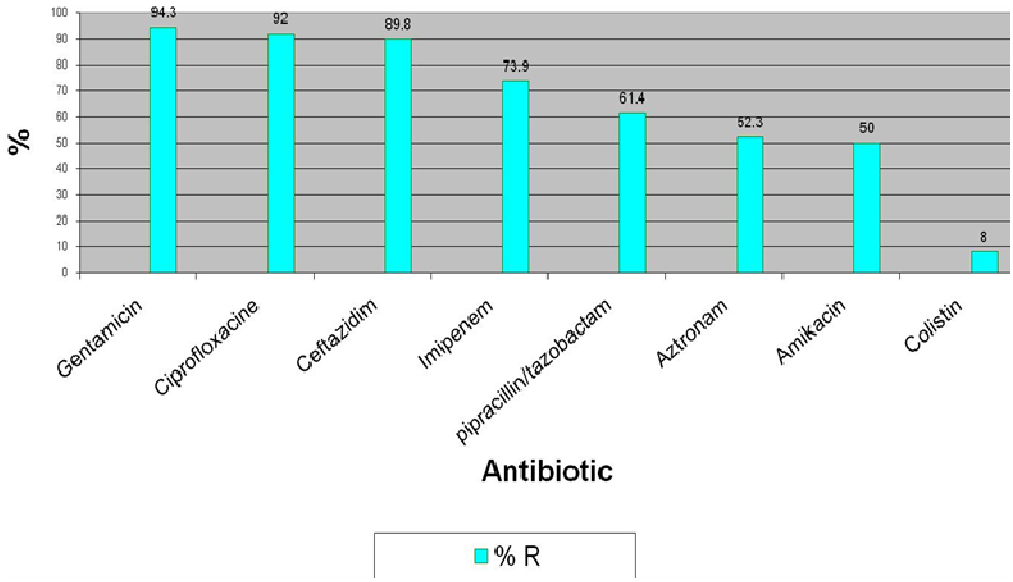


Figure 1. Antibiotic resistance of *P. aeruginosa*.

antibiotic classes used to treat *P. aeruginosa* infections.

MATERIALS AND METHODS

Study location

P. aeruginosa were collected randomly from April 2009 to March 2010 from the University of Malaya Medical Center.

Bacterial isolate

A total of 88 *P. aeruginosa* clinical isolates isolated from various samples collected from different wards of University of Malaya Medical Center. *P. aeruginosa* colonies were identified based on morphology, grams staining, pyocyanin production and biochemical test by API20NE test (bioMérieux, Marcy-l'Etoile, France). According to the manufacturer's instructions using standard laboratory procedures (Osterhout et al., 1991).

Antibiotic susceptibility testing

The susceptibility of various antibiotics against clinical isolates of *P. aeruginosa* was determined using E-test (BIOMERUX) in accordance with the guidelines of the Clinical and Laboratory Standards Institute (Tholen, 2006). *P. aeruginosa* ATCC 27853 were utilized as quality control strains. The antimicrobial tested in this study were pipracilline/tazobactam, ceftazidime, aztreonam, amikacin, gentamicin, ciprofloxacin, imipenem and colistin.

The minimum inhibitory concentration (MIC) for each antibiotic was determined on Mueller- Hinton agar by the E-test® method according to 2006 CLSI, guidelines. Overnight cultures of *P. aeruginosa* on Mueller-Hinton broth were diluted to an initial cell density of 10^7 cfu/ml with fresh Mueller-Hinton broth. Inoculums of 10^5 cfu to achieve a bacterial suspension equivalent to a 0.5 McFarland turbidity standard. Two different E-test® antimicrobial strips placed in opposite gradient directions on Mueller-Hinton agar plate by sterile forceps, printed MIC values are faced upward, [that is, that the bottom surface of the strip containing the antimicrobial gradient is in contact with the agar.

The plates incubated in an inverted position at 37℃ for 20 to 24 h and after incubation. The MICs were read in the intersection point of inhibitory eclipse according to the manufacturer's recommendation.

RESULTS

P. aeruginosa isolated from various clinical samples showed increasing resistance to Gentamicin with 94.3%, followed by (ciprofloxacin) 92%, the (ceftazidime) 89.8%, the (imipenem) 73.9%, pipracilline/tazobactam 61.4%, (aztreonam) 52.3%, and (amikacin) 50% and only susceptible to colistin with 92 % (Figure 1).

Most clinical isolates of *P. aeruginosa* were isolated from urine samples (53.4%), followed by wound (21.5 %), sputum 5.6%, blood 5.6%, tissue 2.2% and 1.13% for other samples (Table 1) Specimens were isolated from different hospital wards. 31 were obtained from surgery ward, 20 from general medicine wards, 13 from orthopedics wards, 7 from paediatric wards, 7 from Neurosurgery wards, 4 from intensive care units, 1 from Cardiac intensive care units, 3 from ENT and 2 from Gynecology wards (Table 2). In the present studies the highest resistant rate of *P. aeruginosa* infections were observed in the surgical department for all antibiotics except colistin that showed the least of resistance followed by the Department of Medicine and Orthopedic (Table 3). The MICs of different antibiotics against *P. aeruginosa* based on the site of the specimen appear highly resistant with urine and wound specimen to be most of the antibiotic except colistin (Table 4).

Table 1. The distribution of *P. aeruginosa* from various clinical specimens.

Source/Site	No of isolates	%
Urine	47	53.4
Wound	19	21.6
Sputum	5	5.6
T/suction	5	5.6
Blood	5	5.7
Tissue	2	2.3
Pus	1	1.1
Lumen tip	1	1.1
Catheter tip	1	1.1
Peritoneal fluid	1	1.1
CSF	1	1.1
Total	88	100

Table 2. Distribution of specimens based on wards.

Wards	Specimen type											
	wound	T/suction	Urine	CSF	sputum	Tissue	Pus	Lumen tip	Catheter	Peritoneal fluid	Blood	Total
Orthopedic	8	0	3	0	0	1	0	0	0	0	1	13
Neurosurgery	1	1	4	0	1	0	0	0	0	0	0	7
Surgery	5	1	20	0	2	1	0	0	1	0	1	31
Paeditric	1	0	3	1	0	0	0	0	0	1	1	7
Gynaecology	0	0	2	0	0	0	0	0	0	0	0	2
Medicine	2	2	12	0	2	0	0	0	0	0	2	20
Cicu	0	1	0	0	0	0	0	0	0	0	0	1
Ent	1	0	1	0	0	0	1	0	0	0	0	3
ICU	1	0	2	0	0	0	0	1	0	0	0	4
Total	19	5	47	1	5	2	1	1	1	1	5	88

DISCUSSION

P. aeruginosa emerged as an important pathogen and responsible for nosocomial infections that is one of the important causes of morbidity and mortality among hospital patients.

In this study, 88 isolates of *P. aeruginosa* from clinical samples received from University of Malaya Medical Centre were studied. These numbers represent the multidrug resistance isolates. The two similar studies, show low rate of resistance in the last three years however this study confirm the increasing rate of resistance rate of colistin (Raja and Singh, 2007; Pathmanathan et al., 2009).

Most of the isolates in current study were collected from urine samples, accounted for 53.4% of the total isolates; this is not surprising as the fact that almost all patients going in for major surgery would be get catheterized. Another study has been shown that the use of indwelling

Table 3. Antibiotic resistance of *P. aeruginosa* based on wards.

Antibiotics		ORTH.	NEURO.	SURG.	PAED.	GYNAE.	MEDIC.	CICU	ENT	ICU	Total
Imipenem	S	4 30.8%	1 14.3%	7 22.6%	2 28.6%	0 0%	6 30 %	0 0.0%	2 66.7%	0 0.00%	22 25.0%
	M	0 0.0%	1 14.3%	0 0.0%	0 0.0%	0 0%	0 0%	0 0.0%	0 0.00%	0 0.0%	1 1.1%
	R	9 69.2%	5 71.4%	24 77.4%	5 71.4%	2 100%	14 70 %	1 100.0%	1 33.3%	4 100.0%	65 73.9%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%
Ceftazidime	S	0 0.0%	0 0.0%	0 0.0%	1 14.3%	0 0.0%	2 10.0%	0 0.0%	1 33.3%	0 0.0%	4 4.5%
	M	1 7.7%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 1.1%
	R	12 92.3%	7 100.0%	31 100.0%	6 85.7%	2 100.0%	18 90.0%	1 100.0%	2 66.7%	4 100.0%	83 94.3%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%
Amikacin	S	0 0.0%	1 14.3%	6 19.4%	1 14.3%	1 50.0%	8 40.0%	1 100.0%	1 33.3%	1 25.0%	20 22.7%
	M	3 23.1%	5 71.4%	11 35.5%	0 0.0%	0 0.0%	3 15.0%	0 0.0%	1 33.3%	1 25.0%	24 27.3%
	R	10 76.9%	1 14.3%	14 45.2%	6 85.7%	1 50.0%	9 45.0%	0 0.0%	1 33.3%	2 50.0%	44 50.0%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%
Pipracilline/ tazobactam	S	4 30.8%	6 85.7%	11 35.5%	0 0.0%	0 0.0%	6 30.0%	0 0.0%	2 66.7%	2 50.0%	31 35.2%
	M	2 15.4%	0 0.0%	1 3.2%	0 0.0%	1 50.0%	1 5.0%	0 0.0%	1 33.3%	0 0.0%	6 6.8%
	R	7 53.8%	1 14.3%	19 61.3%	7 100.0%	1 50.0%	13 65.0%	1 100.0%	0 0.0%	2 50.0%	51 58.0%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%

Table 3. Cont.

Antibiotics		ORTH.	NEURO.	SURG.	PAED.	GYNAE.	MEDIC.	CICU	ENT	ICU	Total
Ciprofloxacin	S	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 5.0%	1 100.0%	0 0.0%	1 25.0%	3 3.4%
	M	1 7.7%	0 0.0%	1 3.2%	1 14.3%	0 0.0%	1 5.0%	0 0.0%	1 33.3%	0 0.0%	5 5.7%
	R	12 92.3%	7 100.0%	30 96.8%	6 85.7%	2 100.0%	18 90.0%	0 0.0%	2 66.7	3 75.0%	80 90.9%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%
Colistin	S	11 84.6%	7 100.0%	28 90.3%	5 71.4%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	81 92.0%
	M	1 7.7%	0 0.0%	2 6.5%	1 14.3%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	4 4.5%
	R	1 7.7%	0 0.0%	1 3.2%	1 14.3%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	3 3.4%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%
Gentamicin	S	0 0.0%	0 0.0%	0 0.0%	0 0.0%	0 0.0%	1 5.0%	1 100.0%	0 0.0%	0 0.0%	2 2.3%
	M	0 0.0%	0 0.0%	0 0.0%	1 14.3%	0 0.0%	1 5.0%	0 0.0%	0 0.0%	1 25.0%	3 3.4%
	R	13 100.0%	7 100.0%	31 100.0%	6 85.7%	2 100.0%	18 90.0%	0 0.0%	3 100.0%	3 75.0%	83 94.3%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%
Aztreonam	S	4 30.8%	1 14.3%	3 9.7%	0 0.0%	0 0.0%	2 10.0%	0 0.0%	2 66.7%	0 0.0%	12 13.6%
	M	2 15.4%	2 28.6%	7 22.6%	5 71.4%	1 50.0%	9 45.0%	0 0.0%	1 33.3%	0 0.0%	27 30.7%
	R	7 53.8%	4 57.1%	21 67.7%	2 28.6%	1 50.0%	9 45.0%	1 100.0%	0 0.0%	4 100.0%	49 55.7%
Total		13 100.0%	7 100.0%	31 100.0%	7 100.0%	2 100.0%	20 100.0%	1 100.0%	3 100.0%	4 100.0%	88 100.0%

The abbreviations ORTH, NEURO, SURG, PAED, GYNAE, MEDIC, CICU, ENT, ICU designated for orthopedic , neurosurgery, surgery ,pediatrics, gynaecologyl, medicinal, cardic intensive care unit, ear nose throat, intensive care unit, respectively.

Table 4. Antibiotic susceptibility pattern of pseudomonas aeruginosa isolates based on site of specimens.

Source	Imipenm	Ceftazidim	Amikacin	Aztronam	Pipracillin/tazobactam	Ciprofloxacin	Colistin	Gentamicin
Urine	S	M	R	S	M	R	S	M
Wound	12	1	34	1	0	46	10	17
Sputum	6	0	13	0	1	18	5	4
T/suction	0	0	5	1	0	4	2	0
Blood	0	0	5	0	0	5	1	2
Tissue	2	0	3	0	0	5	0	0
Pus	1	0	1	0	0	2	0	1
Lumen tip	1	0	0	1	0	0	1	0
Catheter tip	0	0	1	0	0	1	0	0
Peritoneal fluid	0	0	1	0	0	1	0	0
CSF	0	0	1	1	0	0	0	0

catheters lead to an inherent risk for infection (Olayinka et al., 2004).

Isolates collected from wound infections re-present (21.59 %), the wound infections different from patient to patient, in addition from one hospital to another, from one surgical procedure to other (Humphreys, 2009), in many cases hospital patients used broad-spectrum antibiotics as prophylaxis are mostly colonized by *P. aeruginosa* in the lower intestinal tract (Zuanazzi et al., 2010).

Blood and sputum represent 5.6% of the isolates isolated in the present study. This may be referred due to the empirical therapy of *P. aeruginosa* infection and high pressure of this drugs choice, there is a concordance in the highly susceptibility to as the low number of this samples sent in during the study period. Furthermore, *P. aeruginosa* is the main cause for pneumonia and septicemia with attributable deaths reaching 30% in immunocompromised patients (Scarffand and Goldberg, 2008).

The incidence of multidrug resistant *P. aeruginosa* often changeable dramatically between communities, hospitals in the same area and among many patient, populations in the same hospital (Wroblewska et al., 2006; Marcel et al., 2008). This variation faced by physician in the clinical treatment to be responsible for madding clinical management on the antibiotic choice to be effectively and correctly use according to the update data on the prevalence and resistance pattern.

The antibiotic policy guidelines preparation is the need to avoid any bitter experience of misuse of this class of drug therapy, the team efforts of the members comprising consultants, prescribing physicians, and members of health services team should participate in producing an effective plan to make better decisions in this regards (Masood, 2010). In addition, surveillance programs should be conducted periodically to evaluate the sensitivity and susceptibility of these bacteria against prescribed antibiotics. Since the emergence of resistance remains silent for some periods and takes time to become apparent, these antibiotics should be prescribed with cautions so that the bitter experience of antibiotic resistance can be avoided (Jombo et al., 2010). These *in vitro* sensitivity studies also help in cost-effective; prescribing different brands of the antibiotic to the different patients with varying socioeconomic back-grounds (Masood, 2010). Overall, resistance patterns in different regions should be conducted so that the profiles of the specific region are maintained and monitored accordingly. This will therefore act as a guide towards the formulation of a comprehensive monitoring program of chemotherapy.

In conclusion, most of the clinical isolates had a high level of resistance to examined antibiotics except colistin. Inappropriate and incorrect administration of antimicrobial agents in empiric therapies could be one of the reasons of this alarming phenomenon. This problem indicates importance of performing antibiotic susceptibility testing. What is most worrying is the fact that there is a prevalence of a multiresistant phenotype that was only sensitive to colistin. The emergence and rapid spread of multidrug-resistant isolates of *P. aeruginosa* are of great concern worldwide. It is necessary to limit the overuse of antibiotics and to implement a new antibiotic policy and continuous efforts of clinician, microbiologist, pharmacist and community to

promote greater understanding of this problem, better hygiene, postoperative care and management.

ACKNOWLEDGEMENT

We gratefully acknowledge the funding by the University of Malaya (Research grants PPP No. PS184/2010A) and (Research grant /UMRG No. (RG215/10 HTM).

REFERENCES

Babic M, Hujer A, Bonomo R (2006). What's new in antibiotic resistance? Focus on beta-lactamases. Drug. Resist. Upd., 9: 142-156.

Humphreys H (2009). Preventing surgical site infection. Where now? J. Hosp. Infect., 73: 316-322.

Jombo G, Akpan S, Epoke J, Denen A, Odey F (2010). Multidrug resistant *Psudomonas aeruginosa* infections complicating surgical wounds and the potential challenges in managing post-operative wound infections: University of Calabar Teaching Hospital experience. Asi. Pac. J. Trop. Med., 3: 479-482.

Lagamayo E (2008). Antimicrobial resistance in major pathogens of hospital-acquired pneumonia in Asian countries. Am. J. infect. Cont., 36: S101-S108.

Marcel J, Alfa M, Baquero F, Etienne J, Goossens H, Harbarth S, Hryniewicz W, Jarvis W, Kaku M, Leclercq R (2008). Healthcare-associated infections: think globally, act locally. Clin. Microbiol. Infect., 14: 895-907.

Masood S (2010). *In Vitro* Susceptibility Test of Different Clinical Isolates against Ceftriaxone. Oma. Med. J., 25: 199-202.

Olayinka A, Onile B, Olayinka B (2004). Prevalence of multi-drug resistant (mdr) *pseudomonas aeruginosa* isolates in surgical units of ahmadu bello university teaching hospital, zaria, nigeria: an indication for effective control measures. Ann. Afr. Med., 3: 13-16.

Osterhout G, Shull V, Dick J (1991). Identification of clinical isolates of Gram-negative nonfermentative bacteria by an automated cellular fatty acid identification system. J. Clin. Microbiol., 29: 1822-1830.

Pathmanathan S, Samat N, Mohamed R (2009). Antimicrobial susceptibility of clinical isolates of *Pseudomonas aeruginosa* from a Malaysian Hospital. Mal. J. Med. Sci., 16: 28-33.

Raja N, Singh N (2007). Antimicrobial susceptibility pattern of clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital. J. Microbiol. Immunol. Infect., 40: 45-49.

Rubin J, Walker R, Blickenstaff K, Bodeis-Jones S, Zhao S (2008). Antimicrobial resistance and genetic characterization of fluoroquinolone resistance of *Pseudomonas aeruginosa* isolated from canine infections. Vet. Microbiol., 131: 164-172.

Scarff J, Goldberg J (2008). Vaccination against *Pseudomonas aeruginosa* pneumonia in immunocompromised mice. Clin. Vac. Immunol., 15: 367-375.

Strateva T, Yordanov D (2009). *Pseudomonas aeruginosa*-a phenomenon of bacterial resistance. J. Med. Microbiol., 58: 1133-1144.

Tholen D (2006). CLSI evaluation protocols. Med. Lab. Observ., 38: 38.

Wroblewska M, Rudnicka J, Marchel H, Luczak M (2006). Multidrug-resistant bacteria isolated from patients hospitalised in Intensive Care Units. Int. J. Antimicrob. Agen., 27: 285-289.

Zuanazzi D, Souto R, Mattos M, Zuanazzi M, Tura B, Sansone C, Colombo A (2010). Prevalence of potential bacterial respiratory pathogens in the oral cavity of hospitalised individuals. Arch. Oral. Biolo., 55: 21-28.